İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

DEVELOPMENTAL EXPRESSION OF KATANIN p60, p80 AND SPASTIN

M.Sc. Thesis by
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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

GELİŞİMSEL SÜREÇTE KATANİN p60, p80 VE SPASTİN PROTEİNLERİNİN EKSPRESYONLARININ TAYİNİ

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January 2009 Nihan SİVRİ

Molecular Biologist

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ABBREVIATIONS

ADP : Adenosine Diphosphate
ATP : Adenosine Triphosphate
AP : Antibody
BSA : Bovine Serum Albumin
DEPC : Diethyl pyrocarbonate
DIG : Digoxigenin
DNA : Deoxyribonucleic acid
EDTA : Ethylenediaminetetraacetic acid
EtBr : Ethidium bromide
FSP : Familial Spastic Paraplegia
GDP : Guanosine diphosphate
GTP : Guanosine triphosphate
HSP : Hereditary Spastic Paraplegia
IFs : Intermediate filaments
IHC : Immunohistochemistry
ISH : *In situ* hybridization
kDa : kilodalton
min. : minute(s)
mRNA : messenger Ribonucleic acid
MTOC : Microtubule-organizing center
MTs : Microtubules
NTP : Nucleoside Triphosphate
O.C.T. : Tissue freezing medium - Optimum cutting temperature
PBS : Phosphate buffered saline
PFA : Paraformaldehyde
RNA : Ribonucleic acid
RNase : Ribonuclease
SSC : Saline–Sodium Citrate
TAE : Tris-Acetate-EDTA
Tris : Tris(hydroxymethyl)aminomethane
UV : Ultraviolet
V : Volt

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DEVELOPMENTAL EXPRESSION OF KATANIN p60, p80 AND SPASTIN

SUMMARY

Eukaryotic cells organize their microtubule structured cytoskeleton rapidly, during cellular functions such as; cell cycle, growth, differentiation and cell migration. These functions of cells are supplied by dynamics of microtubule network. Evolutionary conserved AAA (ATPases Associated with diverse cellular Activities) family proteins katanin and spastin are microtubule-interacting proteins which are responsible from regulation of cytoskeleton dynamic. Katanin is a heterodimeric protein which consists of 60 and 80 kD subunits. These subunits are named as katanin p60 and katanin p80 according to their molecular weight. It is thought that p60 shows the enzymatic activity with ATP hydolysis and depolymerizes the microtubule, while p80 directs and regulates the activity of katanin. Studies show that two subunits are found with different concentrations in different tissues even in different sites of a neuron. On the other hand, spastin is another microtubule-severing protein which resembles to katanin p60, and is related to hereditary spastic paraplegia.

Aim of the project is to determine microtubule-severing proteins katanin and spastin expression in mitotic and post mitotic tissues at developmental stage. For this purpose, *in situ* hybridization and immunohistochemistry techniques were applied with *Gallus gallus* embryos.

Riboprobes for *in situ* hybridization were produced from the cDNA's of *Gallus gallus* katanin p60, katanin p80 and spastin by transcription with DIG-RNA labelling kit. Sections were taken by cryostat, from the fertilized chicken eggs which were incubated up to 5 and 7 days. *In situ* hybridization and immunohistochemistry were applied for katanin p60, katanin p80 and spastin on sections. Then, samples were visiualized by light microscope.

The results from the *in situ* hybridization indicates the presence of expression of katanin p60, katanin p80 and spastin on spinal cord, somites, notochord, brain and retina. The expression pattern of three proteins are similar, katanin p80 samples show a better colour contrast; so, it is observed that katanin p80 expression is higher on these tissues. While immunohistochemistry application is ineffective for katanin p80 and spastin, it supports the results of *in situ* hybridization for katanin p60.

GELİŞİMSEL SÜREÇTE KATANİN p60, p80 VE SPASTİN PROTEİNLERİNİN EKSPRESYONLARININ TAYİNİ

ÖZET

Ökaryotik hücreler, hücre döngüsü, büyüme, farklılaşma ve hücre göçü gibi faaliyetleri boyunca mikrotubul hücre iskeletlerini hızlı bir şekilde organize ederler. Hücrenin bu gibi fonksiyonları, mikrotubul ağının dinamizmi ile sağlanmaktadır. Evrimsel olarak korunmuş AAA (ATPases Associated with diverse cellular Activities) ATPaz ailesine mensup katanin ve spastin proteinleri hücre iskeletinin düzenlenmesinden sorumlu mikrotubul-ilişkili (microtubul-interacting) proteinler olup, dinamikliğinin oluşması için başlıca öneme sahiptir. Katanin 60 ve 80 kD'luk iki alt üniteden oluşan heterodimerik bir proteindir. Bu alt üniteler büyüklükleri doğrultusunda katanin p60 ve katanin p80 olarak adlandırılmaktadır. p60 alt ünitesinin ATP hidrolizi ile enzimatik aktivite gösteren, mikrotubul depolimerizasyonunu sağlayan parça olduğu, p80'in ise katanine yön veren düzenleyici parça olduğu düşünülmektedir. Yapılan çalışmalar, proteine ait bu iki altünitenin farklı dokularda, hatta nöronun farklı bölgelerinde dahi farklı konsantrasyonlarda bulunabildiğini göstermiştir. Spastin ise, kalıtsal spastik parapleji rahatsızlığı ile ilişkilendirilen ve fiziksel olarak katanin p60 alt ünitesine benzerlik gösteren bir başka mikrotubul kesici proteindir.

Bu çalışmanın amacı, katanin ve spastinin, gelişimsel süreçte, mitotik ve post-mitotik dokulardaki ekspresyonunun RNA ve protein düzeyinde tayin edilmesidir. Bu amaçla *Gallus gallus* embriyosu üzerinde *in situ* hibridizasyon ve buna ek olarak da immunohistokimya uygulamaları yapılmıştır.

In situ hibridizasyon için kullanılacak olan riboproblar, daha önceki çalışmalarda elde edilmiş *Gallus gallus*'a ait katanin p60, katanin p80 ve spastin cDNA'ları üzerinden DIG-RNA labelling kit ile transkripsiyon yapılarak üretildi. Kuluçka makinesinde inkübe edilen döllenmiş tavuk yumurtaları ile 5 ve 7 günlük dönemlerde diseksiyon yapılarak cryostat ile kesit alındı. Kesitlere katanin p60, katanin p80 ve spastin için *in situ* hibridizasyon ve immunohistokimya uygulanıp ışık mikroskobuyla görüntülendi.

In situ hibridizasyon çalışmasından elde edilen sonuçlar katanin p60, p80 ve spastin'in omurilikte, somitlerde, notokordda, beyinde ve retinada eksprese edildiğini göstermektedir. Üç proteinin ekspresyon paterni benzemekle beraber, katanin p80 için elde edilen görüntülerde renk kontrastı daha belirgin olduğundan katanin p80'in ekspresyonunun daha fazla olduğu düşünülmüştür. Immunohistokimya uygulaması katanin p80 ve spastin için sonuç vermezken katanin p60 için *in situ* hibridazyon sonuçlarını doğrular niteliktedir.

1. INTRODUCTION

1.1. Cytoskeleton

Cytoskeleton is the name of the fiber network in the cytosol of eukaryotic cells. This structure is composed from fibrous proteins and it has important roles in some cellular functions such as maintenance of the cell shape, cell motion, cellular division and intra-cellular transport. It supplies a mechanical linkage with other cells and extracellular matrix for the cell [1,2].

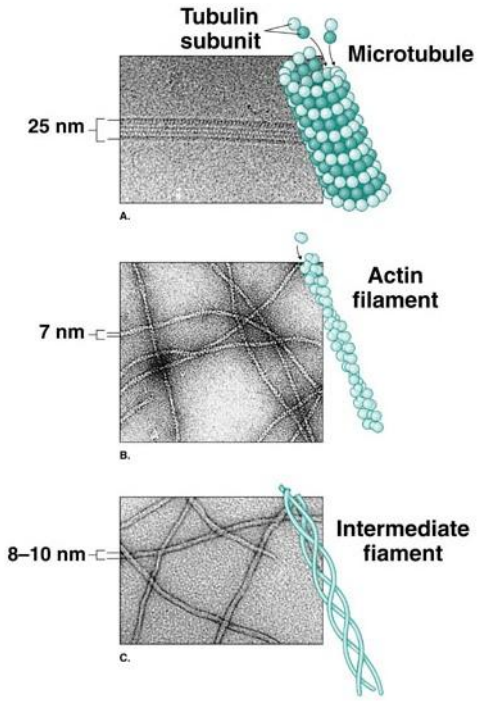

Figure1.1: Filament types of cytoskeleton [3].

Based on their diameters, subunit types and subunit arrangements, cytoskeleton is classified into three types of filaments; microfilaments, intermediate filaments and microtubules.

Microfilaments (actin filaments) are composed from the monomeric actin subunits. They are about 7 nm in diameter and usually attached to the plasma membrane proteins to support the membrane. They have role in the cell movement. The second type, intermediate filaments (IFs), are assembled from lamin subunits. They are

about 10 nm in diameter and serve as a mechanical support for the cell and help the cell to keep its shape. Finally, microtubules (MTs) are assembled from α,β-tubulin subunits. They are about 24 nm in diameter and mainly responsible from the intracellular transport, positioning of the organelles and cell division [1,2].

1.1.1. Microtubules

The longitudinal arrangement of α-β tubulin heterodimers forms protofilaments. 12-15 protofilaments come together through lateral forces and form a 25 nm tube. These highly dynamic cytoskeletal filaments are named as microtubules [4].

A tubulin subunit can bind two GTP molecules. The α-tubulin binds GTP irreversibly and keeps it in non-hydrolyzed form. On the other side, the β-tubulin binds GTP reversibly and hydrolyzes it to GDP after the subunit is added to the microtubule.

As a result of its asymmetric heterodimeric structure, microtubules are polar fibers. This polarity causes different ends for the filament. β-tubulin monomer revealing end is named as the plus (+) end, and oppositely, the other end is the minus (-) one. The assembly and disassembly rates are higher in the plus end rather than the minus one. The subunits are preferentially added to or lost from the plus end of MTs. Polarity of MTs plays role for the direction of vesicle trafficking and the determination of the organelle location [2].

Another tubulin subunit, γ-tubulin, forms a ring complex in the centrosome. This ring serves as a template for the initiation of nucleation. MT elongation starts from this nucleation center. Their plus ends are towards to the cytoplasm of the cell. Therefore, the minus ends of MTs are usually attached to the centrosome in the animal cells, and the centrosome serves as a microtubule-organizing center (MTOC) [5].

MTs act as dynamic railways for the transport of vesicles, organelles, and also chromosomes at mitosis. Motor proteins, like kinesin and dynein, play an important role in these transports by interacting and moving along MTs. MTs help to transport via motor proteins by polymerization and depolymerization [6].

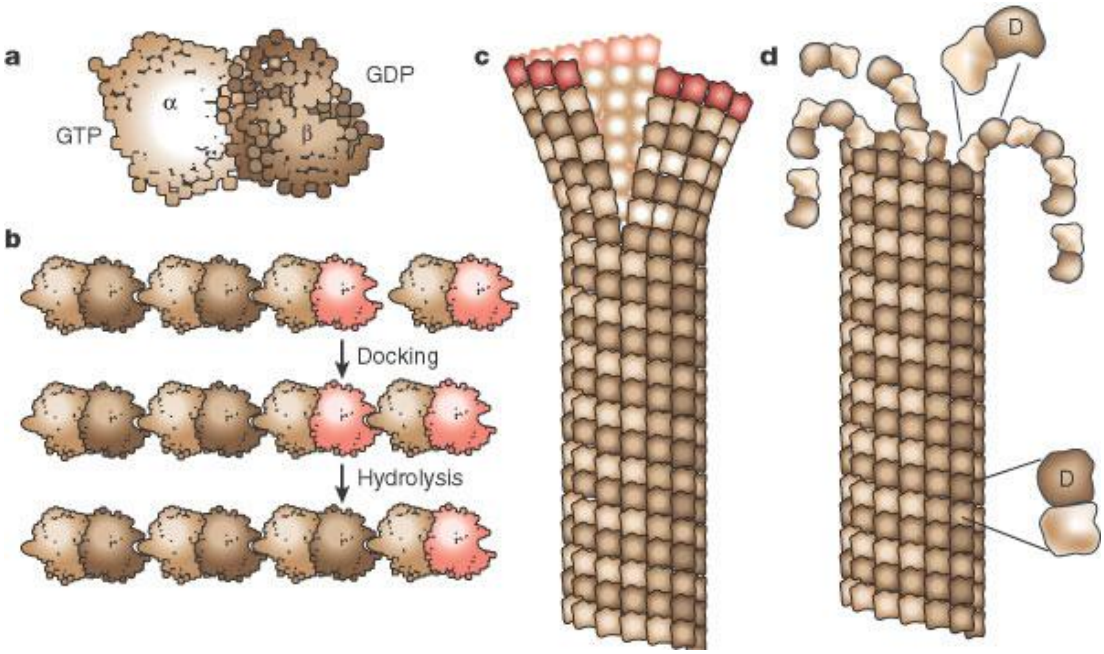

Figure 1.2: MT Structure **a)** α-β tubulin heterodimer **b)** Elongation **c,d)** Shrinking [5]

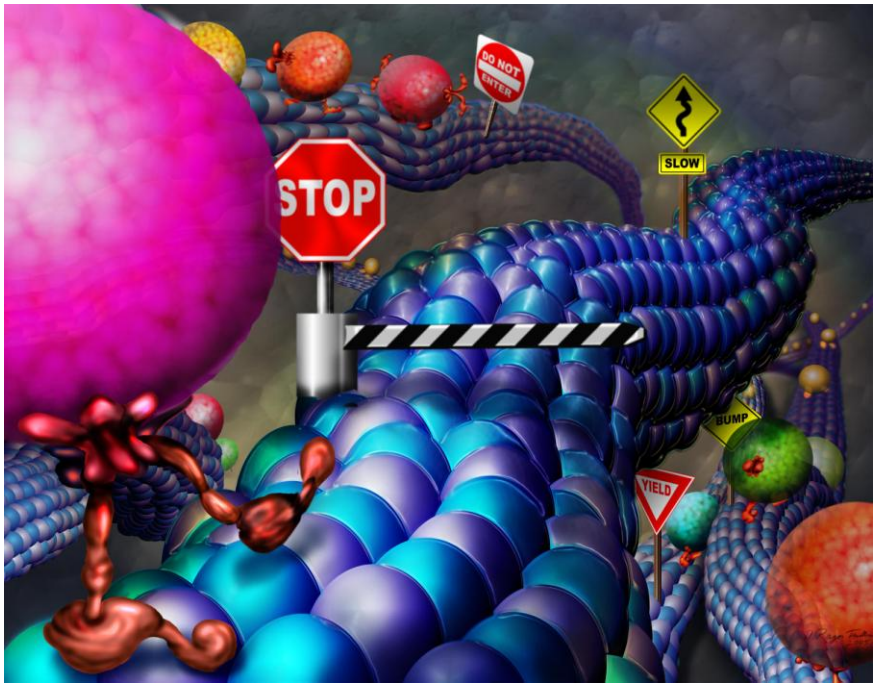

Figure 1.3 : MTs act as railways for the transport by motor proteins. Molecular "signs" on microtubules direct the traffic inside cells [7].

1.1.2. Microtubule Dynamics

Elongation or shrinkage by the addition or loss of tubulin dimers from the ends of the microtubules expose the dynamic characteristic of microtubules. The transformation between slow growth and rapid shrinkage states is known as dynamic instability. This property is forced by the GTPase activity of β-tubulins. GTP hydrolysis starts

when tubulin dimers incorporate into a MT. A conformational change occurs by the conversion of GTP-tubulin to GDP-tubulin. GTP-tubulins on the end supplies a stabilizing cap for MT, if GTP-cap is lost, depolymerization and rapid shrinkage starts [4].

The concentration of the GTP or GDP-bound β tubulins rules the polymerization of MTs. When the concentration of α/β tubulin dimers is higher than the critical concentration (C_c), polymerization of MTs occurs. When the GTP-bound β tubulin concentration is high at the plus end, MTs are kept stable; and when GTP-bound β tubulin concentration is low (GDP-bound β tubulin concentration is high) MTs are unstable and they shrink [2].

Addition of subunits to the plus end while they are being lost from the minus end is another feature of dynamic MTs and named as treadmilling. The speed of lengthening is equal to the speed of shortening in this state [8].

Since many cellular functions, such as cell division and cell differentiation depend on MT dynamics, regulation of MT dynamics is essential in any cell. Also, dynamic behavior of microtubules is thought to be important in the generation and maintenance of neuronal processes, such as dendrites and axons, transport along these structures, as well as the development and maintenance of synaptic transmission in pre- and post-synaptic compartments. Thus, defects in neuronal MT dynamics may cause some human neurological disorders such as Fragile X Syndrome and Hereditary Spastic Paraplegia [9].

There are also some other proteins named microtubule-associated proteins (MAPs) that control the microtubule behaviors. They are tissue and cell type specific. In many different cell types, various MAPs, which have been found to carry out various functions, have been identified. Stabilizing and destabilizing of microtubules, guiding microtubules towards to specific cellular locations, cross-linking microtubules and mediating the interactions of microtubules with other proteins in the cell are the primary functions of these proteins [1].

1.1.3. Microtubule Severing Proteins

Rapid cytoskeleton reorganization need in the cell requires several different mechanisms. In addition to depolymerization, which MTs lose subunits only from

the ends, severing of MTs is observed. There are some proteins which are known as MT severing proteins in the cell [10]. Katanin and spastin are the most well characterized MT severing proteins.

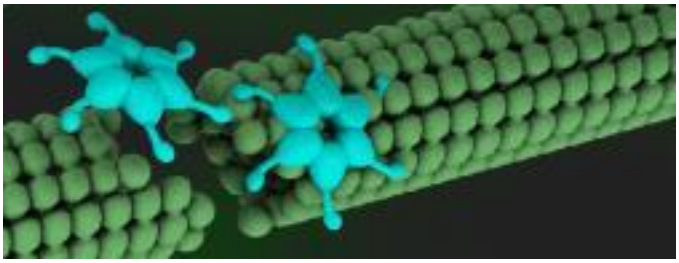

Figure 1.4 : MT severing by proteins [11].

According to the different requirements of the cells, MT severing occurs under different intracellular conditions. Severing may be used for the formation of new small MTs from a long MT in order to generate of new nucleation sites or for the depolymerization of the old filaments. When long MTs are divided into shorter ones, the fluidity of the cell increases [1].

Increasing rate of treadmilling by MT severing is showed in some studies. MT severing is important for releasing the centrosomal MTs. Non-centrosomal MTs are formed in some cell types like neurons, epithelial cells and myocytes by capping the minus ends. Capping of the ends after severing prevents disassembly. These kinds of centrosome-free MTs are important for the growth and maintenance of neuronal processes. In addition, severing of MTs plays a role in decilliation and deflagellation in ciliated or flagellated cells [10].

1.2. AAA Family Proteins

AAA ATPase name represents "***A***TPases ***A***ssociated with diverse cellular ***A***ctivities". They include a group of protein that contains a highly conserved, approximately 220 amino acid residue long ATPase domains, which can be seen in many different cellular activities [12]. They belong to the AAA+ superfamily of ring shaped, P-looped NTPases. Non-ATPase N-terminal domain of the AAA proteins is the primary substrate recognition site. This N-terminal domain is followed by one or two AAA domains [13]. AAA proteins form ring-shaped hexamers with a narrow central pore. The pore motif contains a conserved aromatic residue [14].

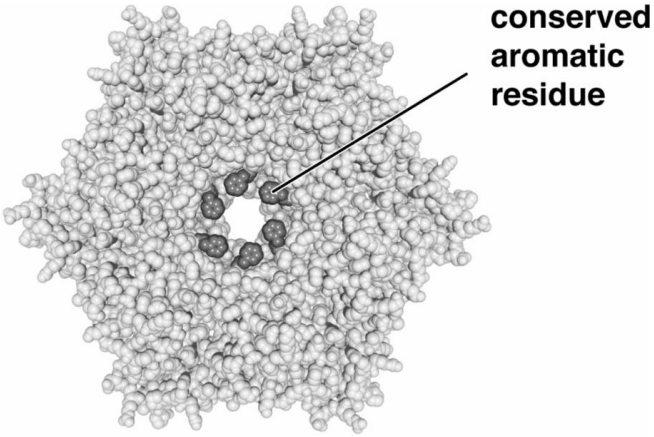

Figure 1.5 : Hexameric ring of the AAA protein. The typical structure of the AAA proteins is the conserved aromatic residue in the middle [14].

These proteins are found in archea, prokaryotes and eukaryotes. Thus, it is thought that they have an ancient and critical function which is evolutionary conserved. They play role in membrane trafficking, cytoskeletal regulation, proteolysis, organelle formation, DNA replication, protein folding, and intracellular motility [12].

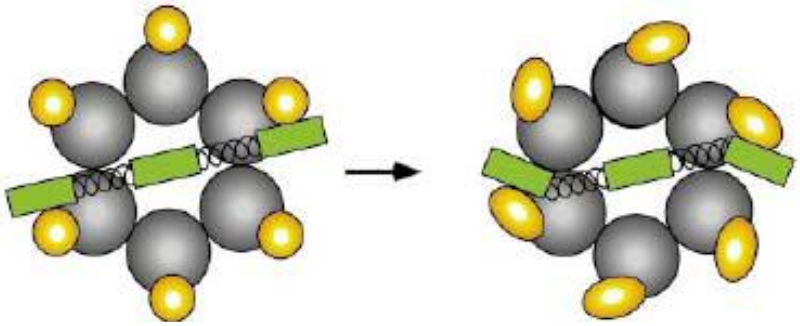

Figure 1.6 : Conformational change of AAA protein ring

The role of AAA proteins depends on ATP hydrolysis. The AAA domains form a hexameric ring and during the ATPase cycle, this ring changes its conformation. For AAA enzyme mechanism, ring-like structures are useful. This structure allows subunits to switch between relaxed and tense states consistently, also supplies structure for binding proteins. Structural rearrengement at the interface region of AAA proteins is triggered by ATP binding. This conformational change increases the interaction between adjacent AAA domains and also the interaction between AAA protein and its target [12].

1.2.1. Katanin

Katanin is a type of AAA ATPase, which breaks the connections between MTs and their nucleating center at the centrosome or MTOCs. It provides the rapid depolimerization of MTs at the poles of mitotic spindle during mitosis. This protein controls the MT dynamics during cell cycle by this way [1,12].

It is the most well characterized microtubule-severing protein. It is originally isolated from sea urchin eggs and it is named as katanin. It is present on various phases at various regions such as centrosome of mitotic, interphase and terminally postmitotic cells, as well as the cytoplasm of many cell types, in all compartments of neurons such as dendrites, axons, growth cones and cell bodies. It is a heterodimeric protein that consists of a p60 subunit (60 kDa) and a p80 subunit (80 kDa). Its p60 subunit is the enzymatic part that severs microtubules, when non-enzymatic p80 subunit has a role in directing katanin p60 to the target and regulating the activity of p60 subunit [8,15].

Studies showed that p60 katanin is able to show ATPase activity and microtubule severing activity in the absence of katanin p80 subunit. The domain which is responsible from binding to microtubules is the p60-N terminal. C-terminal of katanin p60 has great homology with AAA family proteins and it is responsible from the catalytic activity. On the other hand, N-terminal of the p80 subunit has six WD40 repeats. While central domain of katanin p80 has a prolin rich region, C-terminal is responsible from the dimerization with katanin p60 subunit [16].

The concentrations of the katanin subunits may alter in various tissues, at different stages at the development, even in a neuron regionally. *In vitro* studies show that although p60 is able to severe MTs by itself, it works more efficiently in the presence of katanin p80 [17].

Katanin working mechanism is modelled by oligomerizing upon the scaffold. If microtubules are not present at the centrosome, katanin proteins are distributed in the cytoplasm. Katanin-ADP is a monomeric molecule at this state. Katanin molecules and tubulin show affinity to ATP-bound katanin. After katanin changes its ADP for ATP, p60-p60 affinity is triggered and leads the assembly on MT surface.

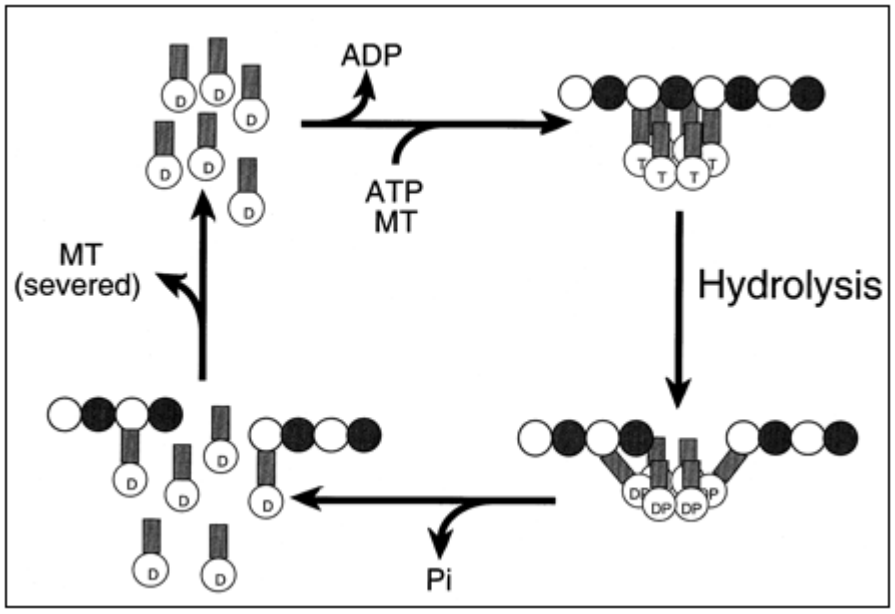

Figure 1.7 : MT severing by katanin model. Only a single protofilament of the microtubule is shown for simplicity. D, DP and T represent ADP, ADP+P_i and ATP states respectively [8].

Assembly of multiple katanin subunits to multiple adjacent tubulin subunits in the microtubule is allowed by oligomerization and 14-16 nanometer katanin ring is formed. ATPase activity starts, after the katanin ring is formed. ATPase reaction causes a phosphate group release and a conformational change occures in katanin. As a result of ATP hydrolysis, tubulin-tubulin contacts destabilize. On the other hand, ADP-bound katanin has a lower affinity for katanin molecules and for tubulin subunits. Then, katanin molecules disassociate and recycle for another severing activity as shown on the figure 1.7 [8]. At low microtubule concentrations (<2 μM), ATPase activity increases with increasing microtubule concentration. On the other hand, at higher microtubule concentrations, ATPase activity decreases until it reaches basal levels [15].

In some cell types, such as neurons and epithelial cells, forming of non-centrosomal MTs by katanin activity is essential. MTs' motility depends on their length. Short MTs have a higher motility than long MTs. In neurons, motion of short MTs is very important for their morphology. Thus, formation of short MTs by katanin activity is curicial [15].

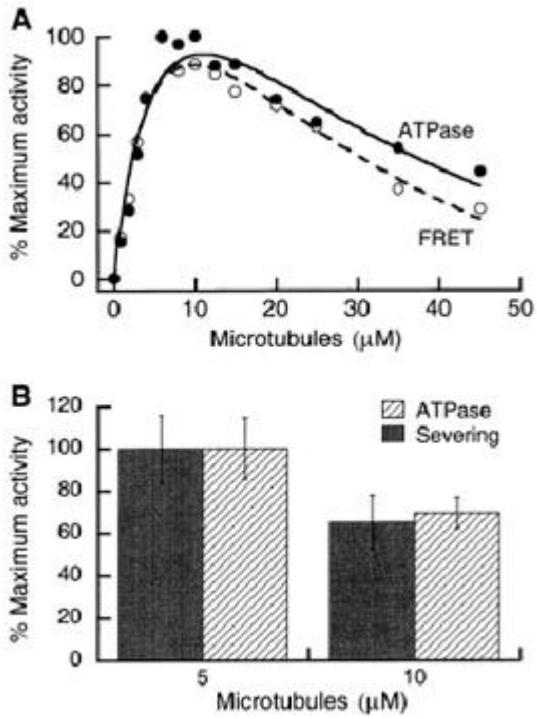

Figure 1.8 : Graphics show the correspondence between katanin ATPase activity, Katanin oligomerization and severing [8].

1.2.2. Spastin

Spastin is a microtubule severing protein which is encoded by the human gene SPAST. Spastin protein is a member of the AAA protein family. Like as katanin and the other members of this protein family, spastin protein has an ATPase domain, too. Spastin has large homology with p60 katanin within AAA domain but their N–terminal regions do not show homology. It is thought as a MT severing protein because of this homology. Cell culture studies put forward that the overexpression of wild type spastin caused the disassembly of MT cytoskeleton and proved the MT severing function of spastin. Studies with overexpression of spastin in muscle of *Drosophila* also proved that spastin is a MT severing protein by deleting their MT networks. It is also showed that spastin has a positive function in carrying on the synaptic growth in *Drosophila* via destabilizing MTs[23,24].

There are two identified transcription variations of this gene which are encoding two alternative isoforms. An autosomal dominant disease, spastic paraplegia is associated with the mutations of this gene [18].

Hereditary spastic paraplegia (HSP), also called familial spastic paraplegias (FSP) or Strumpell-Lorrain disease refers to a group of inherited disorders that are characterized by progressive spasticity in the lower limbs. The disease may also affect sense organs in some types. The disease was described by a German neurologist Adolph Strümpell in 1883. Then, in 1888, a French physician Maurice Lorrain described the disease more detailed [19,20].

Axonal degeneration is the main neuropathological trait of HSP. Degeneration mostly seen in the terminal parts of sensory and motor tracts [20]. No primary demyelination is seen in degenerating neurons. Although anterior spinal horn damage recognized in some cases; dorsal root ganglia, posterior roots and peripheral nerves are found normal. The reason and the mechanism of the disease is still unclear [21]. Although some other genes are identified that are responsible from the hereditary spastic paraplegia, most abundant (~40%) form is related with SPG4 locus mutation that encodes the spastin protein [25].

Errico et al. showed that spastin is not localized in axonal and dendritic processes while mutant spastin amplified in the axons with a study which was done with rat cortical neurons. In the same study, decrease in kinesin staining in mutant spastin overexpressing cells is observed. This occurs to inhibition of organelle transport on cytoskeleton via disruption of MT dynamics by mutant spastin [26].

Another idea about the mutant spastin related neurodegeneration is based on the decreasing amount of short MTs. When mutant spastin prevents MT severing activity, no short MTs can be formed for process generation [15].

SPG4 (SPAST) gene is composed from 17 exons. The protein is 616 amino acids long and ~67.2 kDa. It has two leucine – zipper and coiled – coil dimerization motif [27,28].

Spastin is composed of three domains: 1) a transmembrane TM site on N-terminal 2) a highly conserved microtubule interacting and trafficking domain MIT and 3) ATP binding AAA site [24].

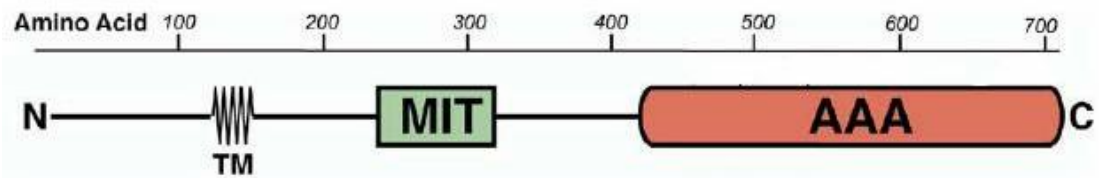

Figure 1.9: Domain localization of spastin.

Localization of spastin is still under discussion. Some studies reported spastin as a cytoplasmic protein, while some studies showed nuclear localization [23] . These findings may be the result of the multifaced cellular role for spastin. In mitotic cells, spastin localizes in the nucleus at the interpase. Then it becomes associated with centrosomes and the spindle MTs during mitosis. In post-mitotic cells, spastin localization becomes discrete in nuclear domain and enriches in the distal axon and in the branching regions such as growth cones. This result gives the idea that spastin controls the MT dynamics in growth cones and manages the stability of axons and axonal transport [29].

1.3. Aim of the Study

Microtubules are essential polymers for the cell. Particularly in neurons, the capacity MT network reconfiguration determines the neuronal morphology. Microtubule severing into shorter pieces is required for the arrangement and transport of MTs. Katanin and spastin are MT severing ATPases that are shown to severe microtubules and play role in generating short microtubules throughout the cell. Also, expression of these two proteins are identified in neurons.

The aim of the study is to determine the expression of microtubule related proteins katanin and spastin in mitotic and post-mitotic tissues at various developmental stages. It is thought that the expression patterns of these two proteins with the same function may be different in various tissues at different embryonic stages. Since their cellular localization in neurons are slightly different, their expression patterns in embryo are thought to be different. Differences between expression patterns and localizations of katanin p60, katanin p80 and spastin may cause variations in process formations.

In order to see the expression patterns of katanin p60, katanin p80 and spastin, *Gallus gallus* was chosen for this study, since it is easier to recieve the animal at

different developmental stages as well as it is a good model organism. It is known that *Gallus gallus* completes organogenesis on the 4th day, so 5 and 7 day old embryos were used for the experiment.

In situ hybridization was used to determine the mRNA expression and immunohistochemistry was performed to confirm the *in situ* hybridization results at the protein level.

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Animals

Fertilized *Gallus gallus* embryos were kindly supplied by PAK TAVUK GIDASAN (Samandıra Köyü, No:54 Kartal-İstanbul, Phone: +90 216 398 48 68).

2.1.2. Laboratory Equipments

Flow hood, autoclave, ice machine, digital scales, microwave oven, electrophoresis system, transulliminator, gel screening system, spectrophotometer, water bath, egg incubator, magnetic stirrer and stirrer bars, pH meter, cryostat, super frost slides, cover slips, vortex, incubator, shaker, block heater, light microscope, petri dishes, falcon tubes, eppendorf tubes, micropipettes and pipette tips were needed during experiments.

Name of the producing companies and the product numbers for the equipments were given on the appendix A.

2.1.3. Chemicals, Antibodies and Kits

Diethyl pyrocarbonate (DEPC), hydrogen peroxide (H_2O_2), ethylenediaminetetraacetic acid (EDTA), sodium hydroxide (NaOH), tris(hydroxymethyl)aminomethane (Tris), (glacial) acetic acid, agarose, ethidium bromide (EtBr), RNA loading dye, RNA ladder marker, phosphate buffered saline (PBS) tablets, paraformaldehyde (PFA), sucrose, sodium chloride (NaCl), sodium citrate dihydrate, hydrochloric acid (HCl), ethanol, tissue freezing medium (optimum cutting temperature-O.C.T.), gelatin, glycerol, resin (mixed bed), formamide, dextran sulfate, Denhardt's solution, salmon sperm DNA, Anti-DIG antibody (Fab Fragments), Bovine Serum Albumin (BSA), NBT/BCIP, Tween 20, Goat serum, Donkey serum, katanin p60 antibody (supplied by Meray Akkor), alkaline

phosphatase conjugated Anti-Mouse antibody, katanin p80 antibody, alkaline phosphatase conjugated Anti-goat antibody, Spastin antibody, alkaline phosphatase conjugated Anti-Rabbit antibody and DIG RNA labeling kit were used during experiments.

Name of the producing companies and the product numbers for the chemicals were given on the appendix B.

2.1.4. Buffers and Other Solutions

2.1.4.1. For Riboprobe Synthesis

2.1.4.1.1. EDTA (0.2 M, pH 8.0, RNase-free)

To prepare 10 ml 0.2 M, pH 8.0 EDTA solution; 0.744 g EDTA was dissolved in 7.5 ml DEPC-treated water. pH was set to 8.0 by adding NaOH. Final volume was brought to 10 ml with DEPC-treated water.

2.1.4.1.2. Tris-Acetate-EDTA (TAE) Buffer (50X, 1X, RNase-free)

To prepare 50X TAE, 242 g Tris base was dissolved in 800 ml DEPC-treated water. 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA was added. pH was set to 8 using HCl. Final volume was brought to 1 L with DEPC-treated water. The solution was kept at room temperature. 1X TAE buffer was obtained from the 50X stock solution. 20 ml 50X TAE was dissolved in 980 ml DEPC-treated water to obtain 1 L 1X TAE buffer.

2.1.4.2. For Preparation of Tissue Sections

2.1.4.2.1. PBS (1X, 100 ml, RNase-free)

1 PBS tablet was dissolved in 100 ml DEPC-treated mQ water using magnetic stirrer. The solution was kept at room temperature.

2.1.4.2.2. PBS (10X, 100 ml, RNase-free)

1 PBS tablet was dissolved in 10 ml DEPC-treated mQ water using magnetic stirrer. The solution was kept at room temperature.

2.1.4.2.3. PFA Solution (4%, 100 ml, RNase-free)

4 g paraformaldehyde, 80 ml DEPC-treated distilled water and 20 µl 10 M NaOH was stirred at 60 ºC until dissolve. 10 ml 10X PBS and DEPC-treated distilled water was added up to volume 100 ml. The solution could be kept at 4 ºC for 2 weeks.

2.1.4.2.4. Sucrose Solution (15%, 100 ml, RNase-free)

15 g sucrose was dissolved in 100 ml 1X PBS using magnetic stirrer. The solution was kept at 4 ºC.

2.1.4.2.5. Sucrose Solution (30%, 100 ml, RNase-free)

30 g sucrose was dissolved in 100 ml 1X PBS using magnetic stirrer. The solution was kept at 4 ºC.

2.1.4.3. Mounting Medium

1 g gelatin, 7 ml glycerol and 6 ml mQ water was stirred with magnetic stirrer at 60°C overnight. The solution was kept at room temperature and heated before each usage.

2.1.4.4. For *In Situ* Hybridization and Staining

2.1.4.4.1. SSC (20X, 2X, 1X, 0.1X, RNase-free)

(20X SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0)

To prepare 50 ml 20X SSC; 8,76 g NaCl and 4,41 g sodium citrate dihydrate was dissolved in 40 ml DEPC-treated water. The pH was adjusted to 7.0 with HCl. Final volume was brought to 50 ml with DEPC-treated water and autoclaved. The solution was kept at room temperature.

Table 2.1 : Dilutions for SSC Solutions.

Dilutions	Final Volume	20X SSC	RNase-free water
2X SSC	50 ml	5 ml	45 ml
1X SSC	50 ml	2,5 ml	47,5 ml
0.1X SSC	50 ml	0.25 ml	49.75 ml

2.1.4.4.2. Deionized Formamide (RNase-free)

15 g mixed bed resin was added into 150 ml formamide. The closed bottle was kept at shaker for 1 hour. The solution was filtered and kept at 4 ºC.

2.1.4.4.3. Prehybridization Buffer (RNase-free)

To prepare 10 ml prehybridization buffer; all ingredients below were added dissolved. The solution was kept at -20 ºC.

Table 2.2 : Ingredients of the Prehybridization Buffer (10 ml).

Chemicals	Amount
20X SSC	2 ml
50X Denhardt's Solution	200 µl
Salmon Sperm DNA	400 µl
Deionized formamide	5 ml
DEPC treated RNAse-free H_2O	2,4 ml

2.1.4.4.4. Hybridization Buffer (RNase-free)

To prepare 10 ml hybridization buffer; all ingredients below were added and dissolved. The solution was kept at -20 ºC.

Table 2.3 : Ingredients of the Hybridization Buffer (10 ml).

Chemicals	Amount
Dextran Sulfate	0.5 g
Deionized formamide	5 ml
20X SSC	2 ml
50X Denhardt's Solution	200 µl
Salmon Sperm DNA	400 µl
DEPC treated RNAse-free H_2O	2,4 ml

Riboprobes for katanin p60, katanin p80 and spastin were dissolved in hybridization solution containing small tubes separately, according to wanted dilutions. 250-400-600-900-1250 ng/ml concentrations were tried for the optimization of hybridization reaction.

2.1.4.4.5. DIG Buffer

(150 mM NaCl, 100 mM Tris-HCl, pH 7.5)

To prepare 50 ml DIG Buffer; 0,44 g NaCl and 2,5 ml 2 M Tris was dissolved in distilled water. pH was set to 7.5 by adding HCl. Final volume was brought to 50 ml with distilled water. The solution was kept at 4 ºC.

2.1.4.4.6. Detection Buffer

(100 mM NaCl, 10 mM Tris-HCl, pH 9.5)

To prepare 50 ml Detection Buffer; 0,29 g NaCl and 0,25 ml 2 M Tris was dissolved in distilled water. pH was set to 9.5 by adding HCl. Final volume was brought to 50 ml with distilled water. The solution was kept at 4 ºC.

2.1.4.4.7. Stop Solution

(10 mM Tris-HCl, 1 mM EDTA, pH 7.5)

To prepare 50 ml Stop Solution; 0.25 ml 2M Tris and 14.61 mg EDTA was dissolved in distilled water. pH was set to 7.5 by adding HCl. Final volume was brought to 50 ml with distilled water. The solution was kept at 4 ºC.

2.1.4.5. For Immunohistochemistry

2.1.4.5.1. PBS-Tween 20 Solution

To prepare 50 ml PBS – Tween 20 solution, 0,25 ml Tween 20 was added in 49,75 ml 1X PBS. The solution was kept at 4 ºC.

2.1.4.5.2. Blocking Solution with Goat Serum

To prepare 10 ml Blocking Solution, 1 ml Goat Serum and 100 mg BSA were dissolved in 1X PBS. Final volume was brought to 10 ml with 1X PBS. The solution was kept at 4 ºC.

2.1.4.5.3. Blocking Solution with Donkey Serum

To prepare 10 ml Blocking Solution, 1 ml Donkey Serum and 100 mg BSA were dissolved in 1X PBS. Final volume was brought to 10 ml with 1X PBS. The solution was kept at 4 ºC.

2.1.4.5.4. Antibody Dilution Solution (Blocking Solution with BSA)

To prepare 10 ml Blocking Solution, 300 mg BSA was dissolved in 1X PBS. Final volume was brought to 10 ml with 1X PBS. The solution was kept at 4 ºC.

2.2. METHODS

2.2.1. Preparation of RNase-free Equipments (DEPC Treatment)

l ml DEPC was added into 1 L mQH_2O in order to make 0.1% DEPC-treated water. All equipments that will be used during experiments, such as falcon tubes, eppendorf tubes, magnetic stirrer bar, dissection equipments etc., were placed in the solution to be RNase free. The box was closed and left overnight on a shaker under the flow hood. Next day, the equipments were removed and autoclaved.

3% H_2O_2 solution was used for the cleaning of laboratory benches and other laboratory equipments that are unsuitable for autoclaving.

2.2.2. Riboprobe Synthesis

DIG RNA Labeling Kit (SP6/T7) was used in order to generate digoxigenin labeled, sense and antisense single stranded RNA probes for katanin p60, katanin p80 and spastin. The following steps were all done with using RNase free materials, in sterile conditions under flow hood and in ice bath.

2.2.1.1. Preparation of Riboprobes by DIG RNA Labeling KIT

1. 7 reactions were needed: katanin p60 sense, katanin p60 antisense, katanin p80 sense, katanin p80 antisense, spastin sense, spastin antisense and control DNA (vial 4 in kit). The probes were synthesized according to the manufacturer's instructions. All reactions were set up at x3 amounts except control, to scale up.
2. Content of a reaction tube was composed from NTP, Buffer, RNase inhibitor, suitable polymerase, template DNA and DEPC treated RNase-free water.

Table 2.4 : Ingredients of the Reaction Tubes.

	p60 sense (EcoRI)	**p60 antisense (XbaI)**	**p80 sense (EcoRI)**	**p80 antisense (XbaI)**	**Spastin (EcoRI)**	**Spastin (XbaI)**	**Control antisense**
NTP (vial 7)	6 µl	6 µl	6 µl	6 µl	6 µl	6 µl	2 µl
Buffer (vial 8)	6 µl	6 µl	6 µl	6 µl	6 µl	6 µl	2 µl
RNase Inhibitor (vial 10)	3 µl	3 µl	3 µl	3 µl	3 µl	3 µl	1 µl
Polymerase T7 (vial 12)	6 µl	-	6 µl	-	-	6 µl	-

Table 2.4 : (Continued) Ingredients of the Reaction Tubes.

Polymerase SP6 (vial 11)	-	6 µl	-	6 µl	6 µl	-	2 µl
Template DNA	13 µl	13 µl	13 µl	13 µl	13 µl	13 µl	4 µl
DEPC treated H_2O	26 µl	26 µl	26 µl	26 µl	26 µl	26 µl	9 µl

3. The tubes were incubated at 37ºC, 2 hours.
4. The reactions were stopped by adding 6 µl (2 µl for the control reaction) 0.2 M EDTA (pH 8.0).
5. 5 µl from the samples were taken for the agarose gel electrophoresis analysis. The rest was kept at –20 °C for later use.

2.2.1.2. Agarose Gel Electrophoresis

In order to prepare 2% 80 ml agarose gel; 1.6 g agarose was dissolved in 80 ml, autoclaved (RNase free) 1X TAE buffer. The mixture was boiled in a microwave oven for 3 minutes, and cooled down. 4 µl EtBr was added into the mixture and mixed gently under the flow hood. The mixture was poured onto the gel plate and the comb was placed. The plate was placed into the electrophoresis tank, after the gel got solidified. The comb was removed.

5 µl from each sample were mixed with 5 µl 2X RNA loading dye particularly. Then, the samples were incubated in 70^oC for 10 minutes. The samples were placed in the ice bath. The samples and the RNA Ladder Marker were loaded on the gel. The gel was run at 100 V for 25 minutes and visualized under UV light to record its photo.

2.2.1.2. Measurement of Probe Concentrations

Obtained riboprobes were diluted 1000 times (1/1000) in RNase-free water in RNase-free tubes. The absorbance values of probes were 260 nm wavelength. Then,

RNA concentrations were calculated by the formula below. The measurements and calculated RNA amounts were given on the table 2.5.

Table 2.5 : Absorbance Values and Concentrations of Riboprobes.

Riboprobe	**Absorbance at 260 nm**	**RNA (µg/ml)**
Control	0,025	1000
Katanin p60 sense	0,032	1280
Katanin p60 antisense	0,024	960
Katanin p80 sense	0,022	880
Katanin p80 antisense	0,013	520
Spastin sense	0,021	840
Spastin antisense	0,040	1600

$$\text{RNA (µg/ml)} = A_{260} \text{ x dilution factor (1000) x 40}$$

Measured riboprobes were allocated and diluted in RNase-free water with 1/10 ratio and stored at -20 °C. Preparations of dilutions were summarized on the table 2.6.

Table 2.6 : Preparation of Riboprobe Dilutions.

Riboprobe	**Stock RNA concentration (µg/ml)**	**Dilution Ratio**	**Stock riboprobe amount (µl)**	**RNase-free water amount (µl)**	**Diluted probe concentration (ng/ µl)**
Katanin p60 sense	1280	1/10	5	45	128
Katanin p60 antisense	960	1/10	5	45	96
Katanin p80 sense	880	1/10	5	45	88
Katanin p80 antisense	520	1/5	10	40	104

Table 2.6 : (Continued) Preparation of Riboprobe Dilutions.

Spastin sense	840	1/10	5	45	84
Spastin antisense	1600	1/10	5	45	160

2.2.3. Incubation of Chicken Eggs

The channels of the egg incubator were filled with water to keep inside of the box humid. The temperature was set at 37.6°C. Then, the fertilized chicken eggs were kept in the adjusted incubator up to 5 and 7 days.

2.2.4. Preparation of Tissue Sections

2.2.4.1. Dissection

The upper side of the egg was marked by pencil before it was taken from the incubator. Then, the egg was wipped by a piece of cotton with alcohol. It was cracked in a petri plate from the marked side. Since the egg was incubated always at the same position, the the embryo was placed in the bottom, and it placed to the upper part after cracking. The embryo was removed from egg's content and washed with PBS.

2.2.4.2. Fixation

The embryo was placed in 4% PFA solution (approximately 10 times amount of the embryo) overnight at 4ºC for fixation. Fixation is essential for RNA retention but overfixation may prevent probe penetration.

2.2.4.3. Hydrostabilizing

The embryo was immersed in 15% sucrose solution (approximately 10 times amount of the embryo) and waited at 4ºC for 2 hours. Then, the embryo was transferred into the 30% sucrose solution approximately 10 times amount of the embryo) and waited at 4ºC overnight, until they sink.

The excess 30% sucrose solution was removed around the embryo by a piece of damp filter paper, before embedding tissues in O.C.T (Optimum Cutting Temperatur Solution).

2.2.4.4. Sectioning

The cryostat was switched on and set to –25 ºC, a night before sectioning, to cool. The embryo was embedded in the O.C.T. in an aluminum foil cup. The cup was placed in the cryostat and waited about 15 minutes until the compound froze completely. 3-5 drops of O.C.T. compound was dropped on the sectioning disc and the aluminum foil cup was placed on it. When the block fixed on the disc, the aluminum foil around the block was ripped off and the specimen disc was inserted in the specimen head. The O.C.T. block was trimmed with sectioning. The thickness was adjusted to 12 nm, and sections were placed on the superfrost slides. The slides were kept in the cryostat after sectioning, and placed in -20 ºC for some weeks.

2.2.5. *In Situ* Hybridization

Serial sections were used to detect the expression of katanin p60, p80 and spastin proteins in each sample by *in situ* hybridization technique.

2.2.5.1. Post-fixation of Tissue Sections for ISH

The slides were removed from freezer and waited until they reach to the room temperature. %4 PFA solution was dropped on each section on the slides and wait about 10 minutes at room temperature. The samples were rinse with 1X PBS, and dried at 37 ºC.

2.2.5.2. Prehybridization

The floor of a wide closed box was covered with filter paper, and the slides were placed on it. Prehybridization buffer was dropped onto samples. Water filled small cups were placed in the box to avoid evaporation and the box was kept at 55 °C for 3 hours.

2.2.5.3. Hybridization

The hybridization buffers (which contain various riboprobes according to the table 2.7.) were taken from -20 °C and denatured at 80 °C for 10 minutes

Table 2.7 : Dilution Amounts of Riboprobes and Preparation of Hybridization Solutions. Hybridization solutions were prepared with 1 ml volume each contains 600 ng riboprobe.

Riboprobe	**Diluted RNA Concentration (ng/µl)**	**Needed amount for 600 ng**
Katanin p60 sense	128	4,7
Katanin p60 antisense	96	6,3
Katanin p80 sense	88	6,8
Katanin p80 antisense	104	5,8
Spastin sense	84	7,1
Spastin antisense	160	3,8

The prehybridization solution was removed from samples and hybridization buffer was added. Hybridization buffer that does not contain any riboprobe was applied on a sample as negative control for staining. Water filled small cups were placed in the box and the box was surrounded by stretch film to avoid evaporation. The box was kept in 55 °C incubator overnight for hybridization.

The next day, the hybridization solutions were removed from the samples and 2X SSC was applied for 15 minutes at 55 °C. 2X SSC was changed with 1X SSC and samples were kept at 55 °C for 15 minutes. After 15 minutes, 1X SSC was renewed and the samples were kept at 55 °C for 15 minutes. Then, 1X SSC was removed and 0.1X SSC was applied for 15 minutes at 55 °C. There is no need to be RNase-free after these steps.

2.2.5.4. Staining

1X SSC was removed and DIG Buffer was applied for 10 minutes at room temperature. After DIG Buffer was removed, 1% BSA in DIG buffer was droppedon

the samples and the slides were kept at room temperature for 60 minutes. Anti-DIG AP was diluted into 1% BSA + DIG Buffer solution at 1/1000 ratio. 1% BSA in DIG Buffer solution was removed from the samples and the diluted Anti-DIG AP solution was dropped on them. The slides were kept at room temperature overnight.

2.2.5.5. Detection

The next day, AP solution was removed from the samples. DIG Buffer was applied for 30 minutes at room temperature. DIG Buffer was changed and the samples were waited for 30 minutes at room temperature again. After the DIG Buffer was removed, Detection Buffer was applied for 10 minutes at room temperature.

100 µl NBT/BCIP was diluted in 5 ml Detection buffer. Diluted NBT/BCIP was added onto samples and the samples were kept at room temperature, at dark, for 2 hours. After the NBT/BCIP solution was removed, Stop Solution was applied for 1 hour at room temperature. Stop Solution was removed and the slides were washed by dipping them in sterile water 2 times. The slides were kept at 37 °C for 10 minutes to dry. 20 µl heated mounting medium was dropped onto cleaned coverslips. Then, the coverslips were placed on the slides. The samples were kept at room temperature until microscopy.

2.2.6. Immohistochemistry

Serial sections were used to detect the expression of katanin p60, p80 and spastin proteins in each sample by immunohistochemistry technique.

2.2.6.1. Post-fixation of Tissue Sections for IHC

Sections were removed from the freezer and waited about 20 minutes until they reach to the room temperature. Cooled methanol (methanol kept at -20 ºC) was dropped on each section on the slides and the slides were kept at -20 ºC for 5 minutes. The excess alcohol was dried on the slides and the samples were airdried by waiting about 30 minutes at room temperature.

2.2.6.2. Immunoenzyme (AP) Staining

After the samples were washed with 1X PBS, PBS-Tween 20 solution was added on the samples and kept for 2 minutes. This step was repeated with fresh solution again. The sections were incubated with suitable blocking solutions (given on the table 2.8.) for 30 minutes to block non-specific binding of immunoglobulin.

Primary antibodies were diluted according to the appropriate values on the table 2.8. with antibody dilution solution. Then, the solutions were applied on the samples overnight at +4 °C.

Table 2.8 : Apropriate Blocking Solutions, Primary and Secondry Antibodies and Their Dilutions According to the Proteins.

	Katanin p60	**Katanin p80**	**Spastin**
Blocking Solution	With Goat Serum	With Donkey Serum	With Goat Serum
Primary AP	p60 AP, Mouse	p80 AP, Goat	Spastin AP, Rabbit
Dilution of 1. AP	2/100 – 5/100	1/50	1/500
Secondary AP	Anti-Mouse IgG - Alkaline Phosphatase	Anti-Goat IgG – Alkaline Phosphatase	Anti-Rabbit IgG - Alkaline Phosphatase
Dilution of 2. AP	1/500	1/500	1/500

No primary antibody was applied on some sections in order to be used as negative control. These samples were treated with blocking solution with goat or donkey serum and then, incubated with AP dilution solution without AP. Organization of the negative controls were summarized on the table 2.9.

The next day, the sections were rinsed in PBS-Tween 20 solution for 2 minutes, 2 times. Secondary antibodies were diluted in AP dilution solution and applied on the samples for 2 hours at room temperature. Then, the sections were rinsed in PBS-Tween 20 solution for 2 minutes, 2 times again.

Table 2.9 : Organization of Negative Controls.

Treatment	N.C. Type I	N.C. Type II	N.C. Type III	N.C. Type IV	N.C. Type V
Donkey Serum	+	-	+	-	-
Goat Serum	-	+	-	+	-
2.AP Donkey Anti-goat	+	-	-	-	-
2.AP Goat Anti-mouse	-	+	-	-	-
NBT/BCIP	+	+	+	+	+

2.2.6.3. Detection

60 µl NBT/BCIP was diluted in 3 ml detection buffer. The samples were incubated with this solution for 30 minutes at room temperature in dark. Then, the sections were rinsed with distilled water for 2 minutes, 2 times. After the samples were airdried, 20 µl heated mounting medium (prepared before) were dropped on the samples and cleaned coverslips were placed on the slides.

2.2.7. Microscopy

Samples were observed with Leica DM2500 light microscope and the images were recorded with the consistent software.

3. RESULTS AND DISCUSSION

3.1. Riboprobe Synthesis

In order to generate digoxigenin labeled, sense and antisense riboprobes for katanin p60, katanin p80 and spastin; DIG RNA Labeling Kit (SP6/T7) was used. This kit uses linearized DNA as a template and RNA polymerase to produce DIG-labeled and single-stranded RNA probes for any hybridization. Transcription occurs in vitro and needs digoxigenin-UTP for labelling. Then, DIG-labeled RNA probes can be detected with anti-digoxigenin conjugated alkaline phosphatase. The bound antibody conjugate is then visualized with the chemiluminescent substrate NBT/BCIP.

All steps were applied under sterile and RNase free conditions to avoid from contamination. 1 μg of purified DNA templates (linearized 180bp-ch-p60-katanin-pSPT19 plasmid, linearized 180bp-ch-p80-katanin-pSPT19 plasmid and linearized 180bp-ch-spastin-pSPT19 plasmid) were used for RNA labeling reaction. All other contents of the reaction (NTP, Buffer, RNase inhibitor, polymerase, and DEPC treated RNase-free water) were set up at x3 amounts except control reaction, to scale up. were performed by DIG RNA labeling kit.

In Figure 3.1 lane 1 shows the synthesis of DIG-labeled antisense Neo transcripts of 760 bases in length. The DNA template used for the synthesis of this reaction was 0.25 mg/ml control DNA 2 pSPT19-Neo supplied by the DIG RNA labeling kit (vial 4). A very dense band seems between 500 bases - 1000 bases, which points out the 760 bp labeled RNA product. Since DNase treatment was not performed, the reaction mixture contains DNA fragments of different sizes and there are some faint bands.

Lane 2, 3 and 4 show the sense riboprobes of katanin p60, katanin p80 and spastin sequentially. The bands in these lanes are approximately 200 bases in length, verifying the correct transcription of 180 bp region.

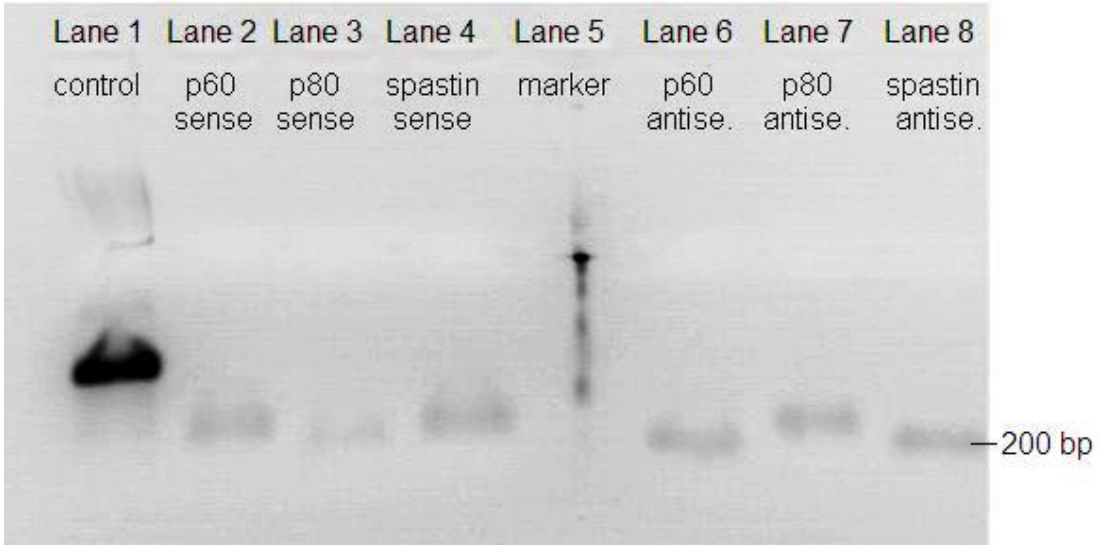

Figure 3.1 : Agarose gel electrophoresis results of DIG-labeled products 1) Control DNA (vial 4) 2) Katanin p60 sense riboprobe 3) Katanin p80 sense riboprobe 4) Spastin sense riboprobe 5) RNA marker (Fermentas) 6) Katanin p60 antisense riboprobe 7) Katanin p80 antisense riboprobe 8) Spastin antisense riboprobe

Conformably, lane 6, 7 and 8 show the antisense riboprobes of katanin p60, katanin p80 and spastin sequentially. The bands in these lanes are approximately 200 bases in length, verifying the correct transcription of 180 bp region as the same with lane 2, 3 and 4.

Table 3.1 : Concentrations of Produced Riboprobes.

Riboprobe	**RNA (µg/ml)**
Control	1000
Katanin p60 sense	1280
Katanin p60 antisense	960
Katanin p80 sense	880
Katanin p80 antisense	520
Spastin sense	840
Spastin antisense	1600

Scaling up was purposed in this step by setting up at x3 amounts of ingredients in the reactions tubes except control sample. However, scaling up reaction seems to be unsuccesful since RNA concentrations of the scaled up reactions are not higher than the control reaction. Even, katanin p60 antisense, katanin p80 sense, katanin p80

antisense and spastin sense concentrations are lower than control. Table 3.1 indicates the concentration results that are calculated according to the absorbance measurement as explained in the methods part 2.2.1.2.

3.2. In Situ Hybridization

To optimize the hybridization conditions 45°C and 55°C temperatures, 400, 600 and 900 ng/µl probe concentrations were tried in the first *in situ* hybridization experiment. According to the preliminary results, the temperature was set as 55°C, the riboprobe concentration was set as 600 for the following experiments. Also blocking for the staining step was optimized as 1% BSA in DIG buffer.

When in situ hybridization was applied with the same concentration of riboprobes for three proteins, it seemed that colour contrast for katanin p60 and spastin is lower than the colour contrast of katanin p80. Katanin p80 hybridization always gave the best colour contrast, even it was used with 250 ng/µl concentration when spastin and katanin p60 used with 1250 ng/µl concentration (see figure 3.2). Thus, it can be thought as katanin p80 expression is much more than katanin p60 and spastin expression. On the other hand, adenine numbers of the template DNAs are not equal for katanin p60, katanin p80 and spastin. Since the DIG RNA labeling KIT places the DIG labelled UTPs and non-labelled UTPs randomly to the opposite of adenines on the template DNA, and staining of the tissues are proportional with the DIG amount in the probe, strong colour contrast may also be as a result of higher DIG labelled uracil amount. Ultimately, ISH is not a quantitative method.

Although more embryos were used for the experiment, just 2 of them have optimized and comperatible results. ISH results obtained from the 5 day old embryo came from 3 different sets of experiment and the results from the 7 day old one came from 2 sets of experiment.

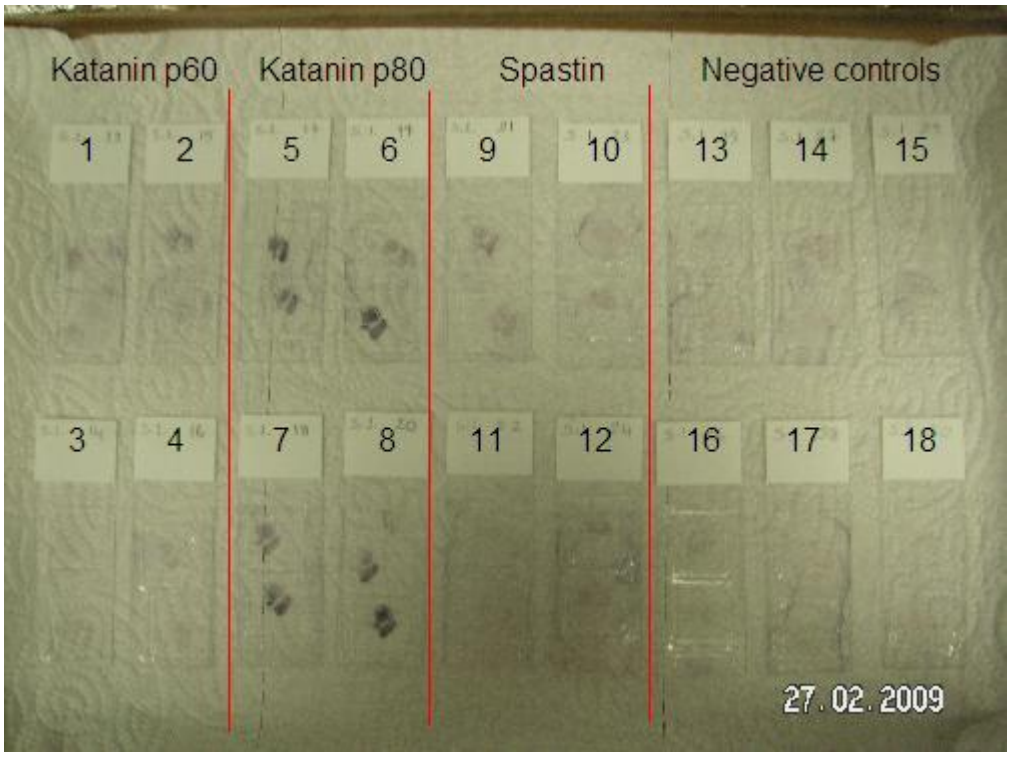

Figure 3.2 : Comparision of colour contrasts for three proteins. First four slides (sample 1, 2, 3 and 4) are the results of katanin p60 *in situ* hybridization with 1250 ng/µl concentrated probes. Next four slides (sample 5, 6, 7 and 8) are the results of katanin p80 *in situ* hybridization with 250 ng/µl concentrated probes. Then, next four slides (sample 9, 10, 11 and 12) are the results of spastin *in situ* hybridization with 1250 ng/µl concentrated probes. The last six slides (sample 13, 14, 15, 16, 17 and 18) are negative controls.

When tissues were examined under light microscope, it seemed as all three proteins, katanin p60, katanin p80 and spastin, have expression in all tissues except blood vessels. Colour contrast at spinal cord, notochord, somites, neural retina and brain is high. Expression pattern of all three proteins seem similar. It can be said that these three proteins are expressed in the same tissues. Results are consistent at 5 day old and 7 day old embryos. The figures below show the microscopic images of ISH results.

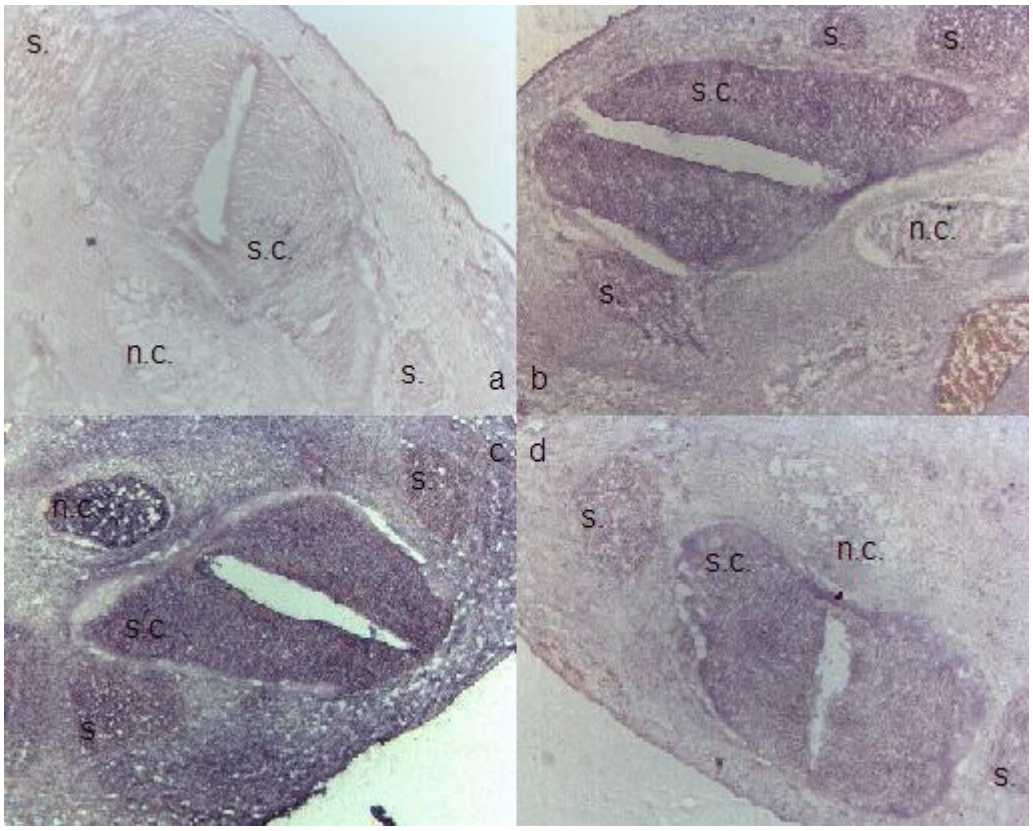

Figure 3.3 : ISH results with x5 magnification, showing the spinal cord and surrounding area of *Gallus gallus* embryo. a) Negative control, b) Katanin p60, c) Katanin p80 d) Spastin. "s.c.", "n.c". and "s." represent spinal cord, notochord and somite, respectively. Although general expression patterns are similar for katanin p60, katanin p80 and spastin, expression at notochord show difference (See also figure 3.5).

Images of the negative controls for ISH were scored "0" as a start point, and katanin p80 samples were scored with "+3" showing as maximum staining. Katanin p60 and spastin results can be scored "+2" and "+1" respectively. However, the expression levels change from tissue to tissue to another on the same sample within itself.

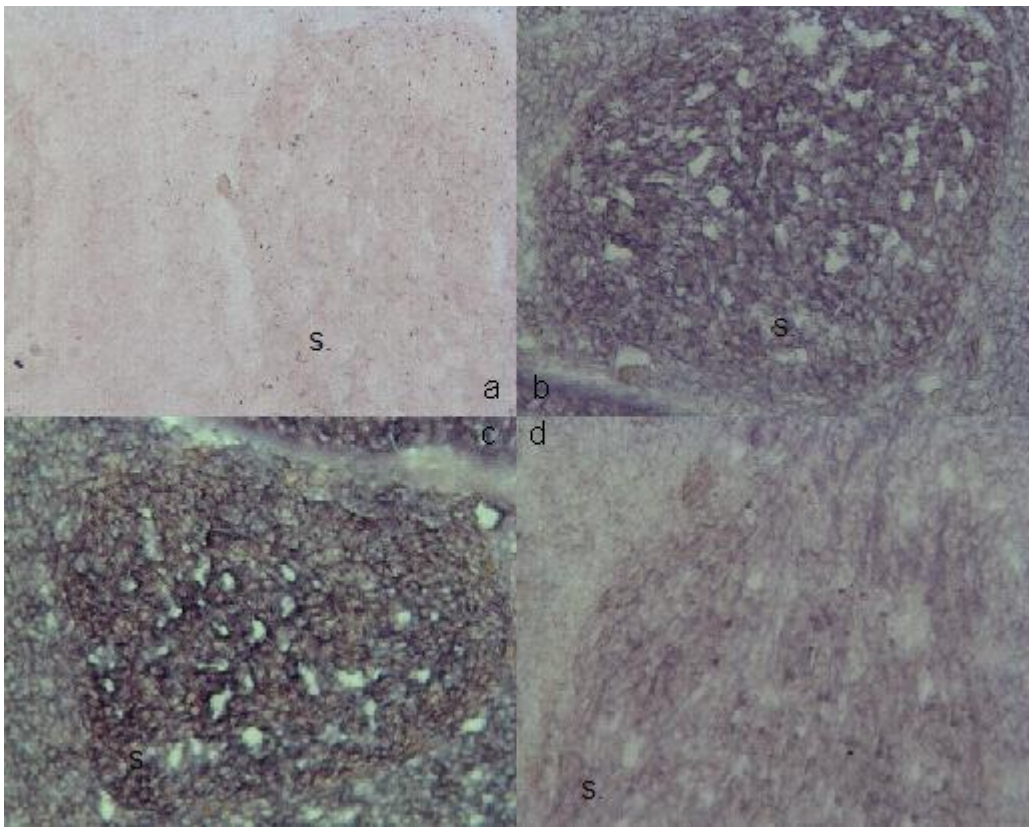

Figure 3.4 : ISH results with x20 magnification, showing the somites of *Gallus gallus* embryo. a) Negative control, b) Katanin p60, c) Katanin p80 d) Spastin. Darker area shows somites. "s." represents the term somite.

The expression levels on somite region for four images; negative control was scored "0", katanin p60 "+2", katanin p80 "+3" and spastin "+1".

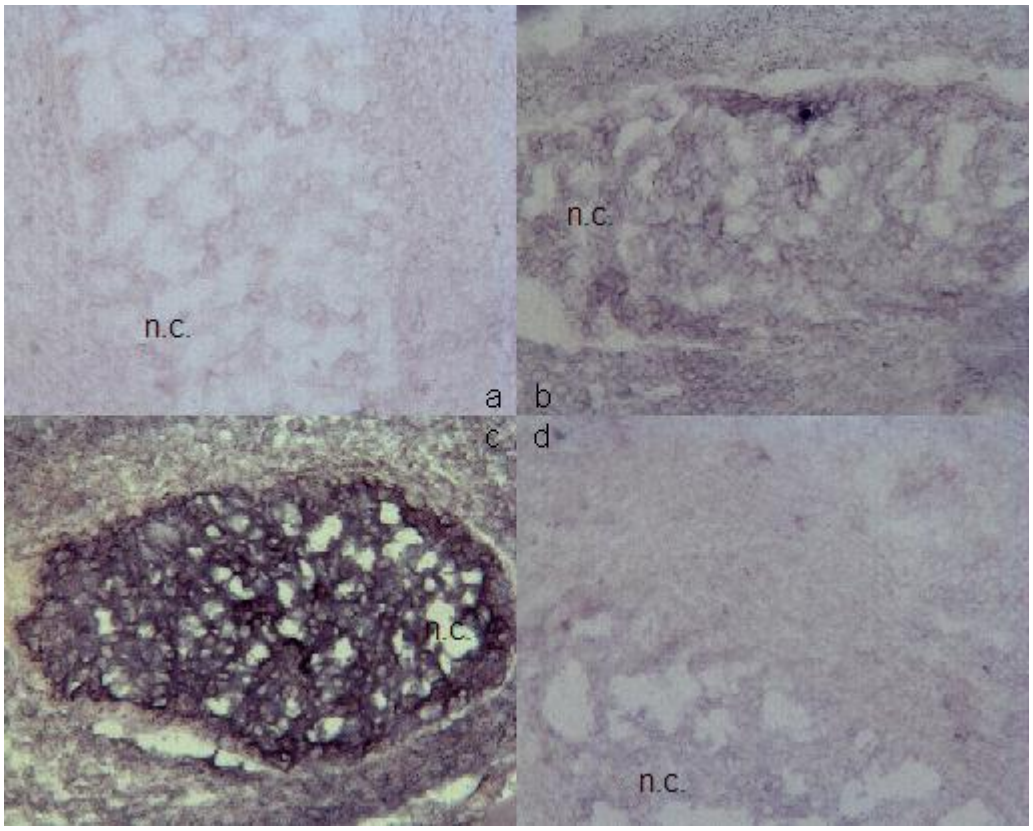

Figure 3.5 : ISH results with x20 magnification, showing the notochord of *Gallus gallus* embryo. a) Negative control, b) Katanin p60, c) Katanin p80 d) Spastin. The darker, elliptical area shows the notochord. "n.c." represents notochord.

Colour contrast at notochord is different in samples. It is thought that katanin p80 expression on notochord is strong, while katanin p60 expression is weak at the same area (See also figure 3.3). The expression levels on notochord region for four images; negative control was scored "0", katanin p60 "+1", katanin p80 "+3" and spastin "0". Spastin expression seems negligible for this tissue.

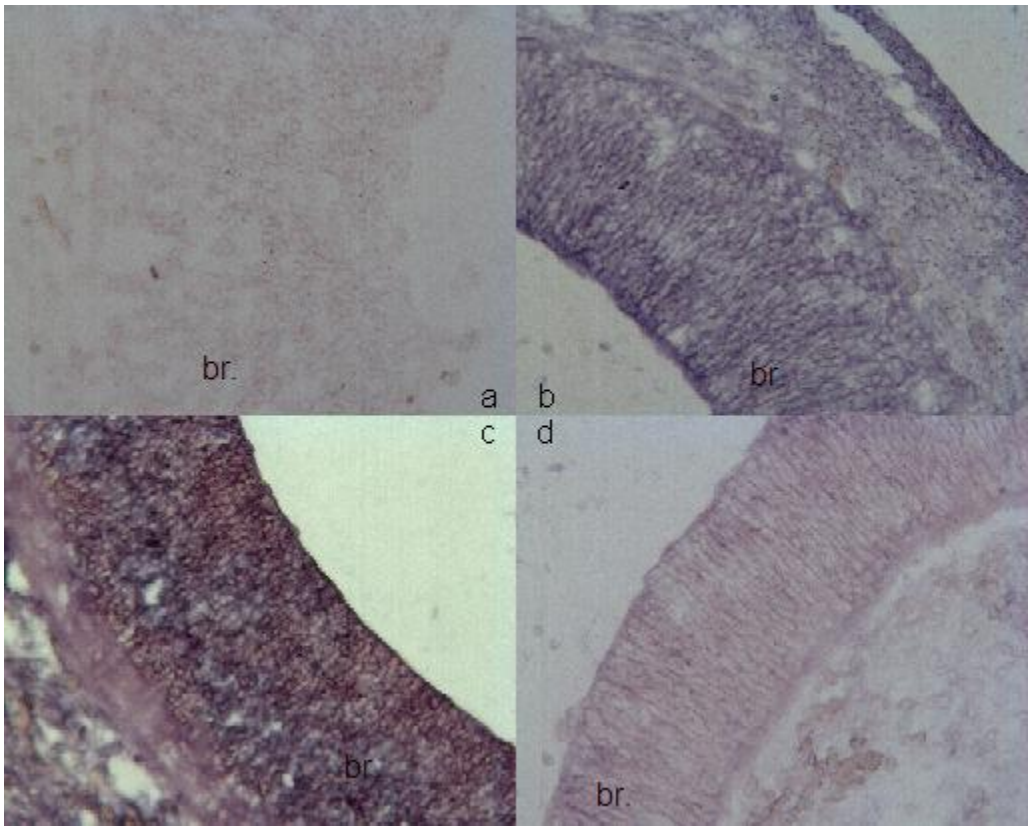

Figure 3.6 : ISH results with x20 magnification, showing the brain of *Gallus gallus* embryo. a) Negative control, b) Katanin p60, c) Katanin p80 d) Spastin. "br." represents midbrain. Darker area which seems as a thick line on the edge shows the midbrain. White empty spaces show the midbrain cavity of the embryo.

The expression levels on brain for four images; negative control was scored "0", katanin p60 "+2", katanin p80 "+3" and spastin "+1".

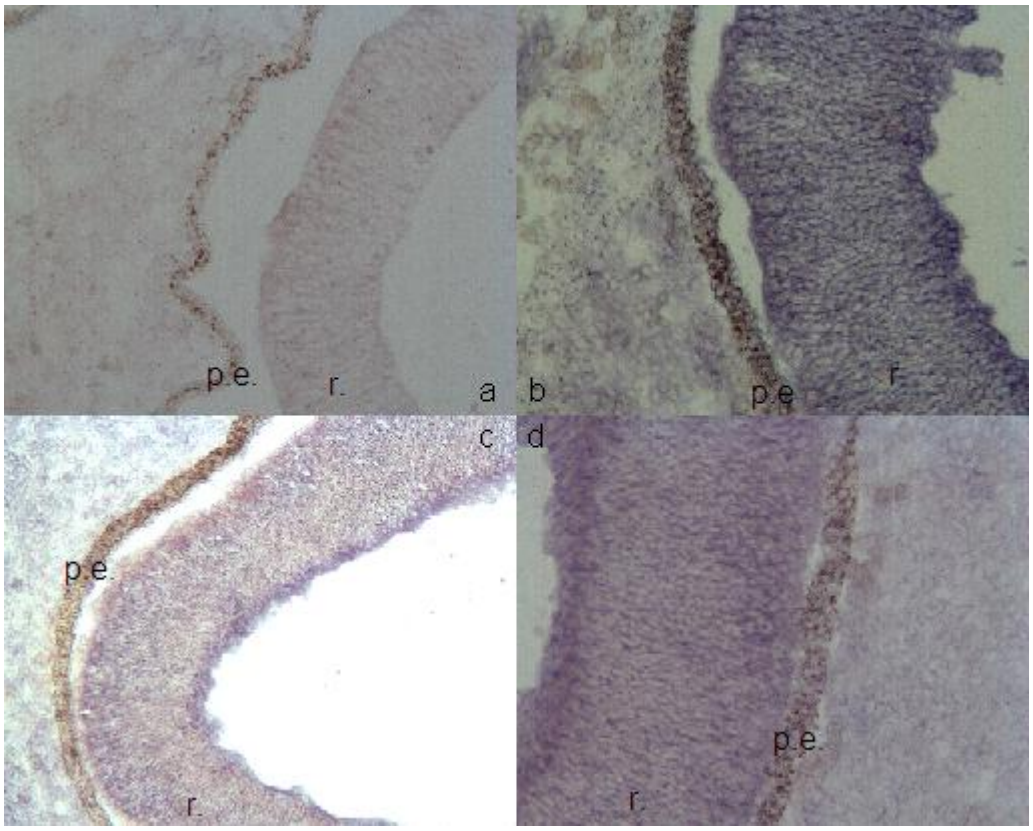

Figure 3.7 : ISH results with x20 magnification, showing the retina of *Gallus gallus* embryo. Pigment epithelium is colourful itself without any staining. a) Negative control, b) Katanin p60, c) Katanin p80 d) Spastin. The right side of the figure a, b, c and the left side of the figure d show sensory retina with optic nerve fibre layer and young retinal ganglion cells. The darker, brownish lines in the middle show the retinal pigment epithelial layers. Sclera and choroid place on the other side of pigment epithelium. "r." and "p.e." refer to retina and pigment epithelium, respectively.

The expression levels on retina for four images; negative control was scored "0", katanin p60 "+3", katanin p80 and spastin "+2".

When ISH experiment was repeated all results were likewise. At start, colour contrast in all tissues were commented as background. However when this staining was not eliminated in other optimized experiments, it was commented as expression in all tissue types. Still, expression on specific tissues such as spinal cord, notochord, somites, brain and retina as mentioned before, is stronger. Only blood tissue is out of expression.

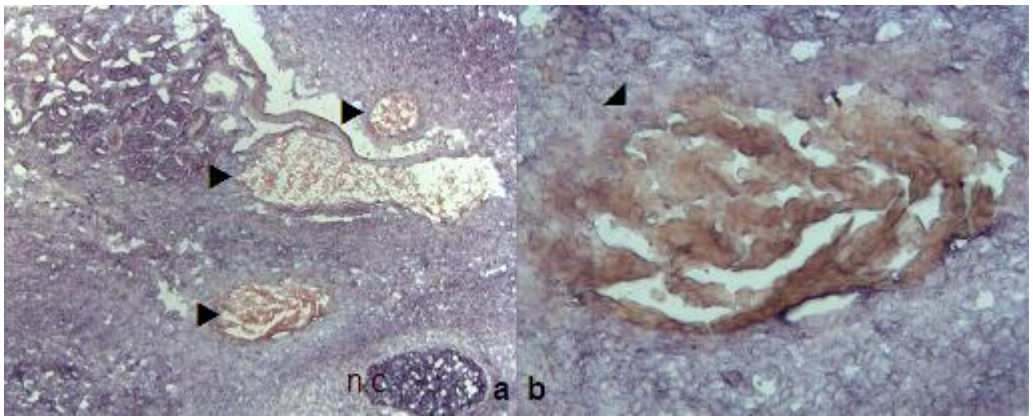

Figure 3.8 : ISH results for katanin p80 expression. No expression seems at blood tissue. a) Blood tissue area with x5 magnification, b) The same area with x20 magnification. Black arrows show the blood tissues, "n.c." refers to notochord.

Negative controls always seem unstained in each experiment set. This result supports the staining specifity of alkaline phosphatase.

3.3. Immunohistochemistry

Immunohistochemistry was applied three times on each 5 and 7 days old two different embryos. Since there is not any katanin antibody for chicken, no katanin p80 antibody was used for the first time, but it is used on the other experiment sets. On the other hand, used katanin p60 antibodies, were produced against mouse katanin p60 in Dr. Karabay's lab. Nevertheless they showed cross-reaction with chicken katanin, too.

When the experiment repeated, katanin p80 antibody (produced against human katanin p80) was used for IHC besides the other antibodies. Katanin p60 and katanin p80 antibodies were never tried on chicken tissues before. The results are positive for katanin p60, but there are no results for immunohistochemistry of katanin p80. Although the sequence of human katanin p80 and chicken katanin p80 have similar regions, the antibody did not give cross-reaction on chicken samples. The experiment was repeated with higher antibody concentration but no significant results were obtained.

IHC results for spastin are also negative although it was repeated with higher antibody concentrations, too. This may be as a result of unoptimized conditions or eventhough the datasheet of the antibody from the manufacturer indicates that there

is a cross-reaction with chicken, this information is not supported with the evidence by the manufacturer.

In summary, IHC for katanin p80 and spastin are not conclusive. The samples after IHC reaction seemed the same with negative controls. On the other hand, katanin p60 antibody staining gave best colour contrast at brain, retina, spinal cord and somites. Notochord was not detected because of the sample orientation during sectioning. Expression pattern seems to be in accordance with the ISH results.

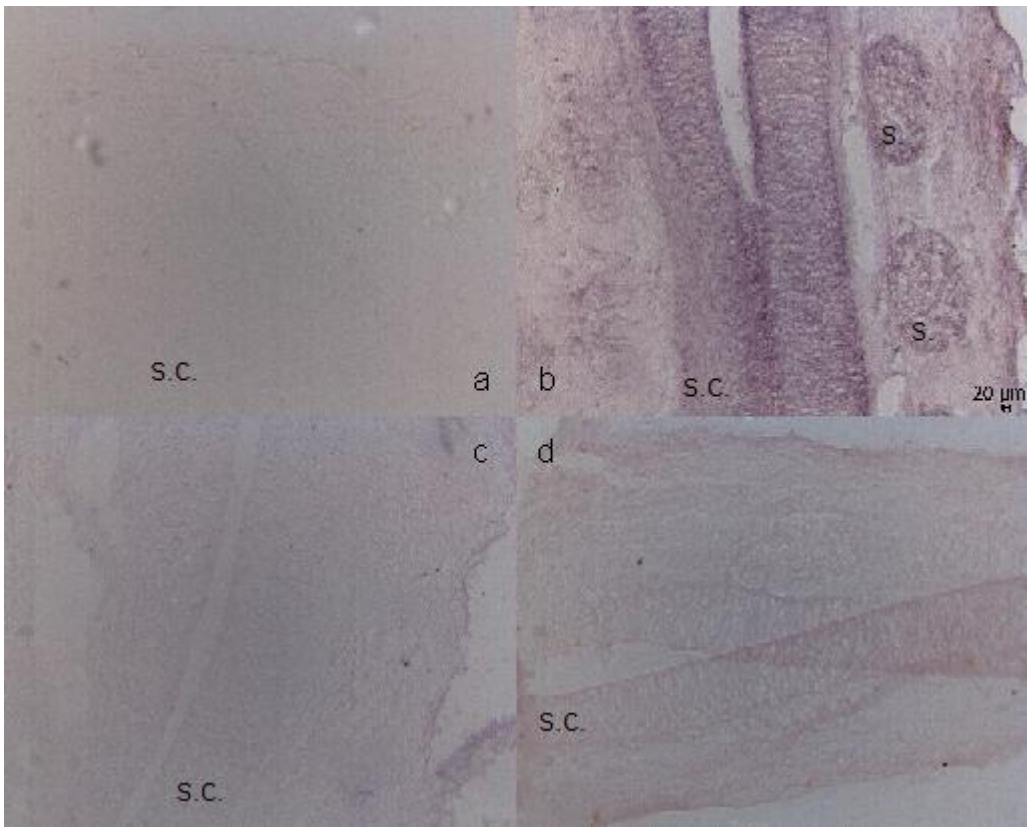

Figure 3.9 : IHC results with x5 magnification, showing the spinal cord and somites of *Gallus gallus* embryo. a) Negative control, b) Katanin p60, c) Katanin p80 d) Spastin. Tissue is folded on the sample d. "s.c." and "s." refer to spinal cord and somite, respectively.

Images of the negative controls for IHC were scored "0" as a start point, and katanin p60 samples for brain and retina were scored with "+3" showing as maximum staining. Scoring for katanin p80 and spastin results are not applicable since the immunohistochemistry experiment was ineffective for these two proteins.

The expression levels on spinal cord - somite region for four images; negative control was scored "0", while katanin p60 was "+2". Katanin p80 and spastin are inconclusive (see figure 3.9.).

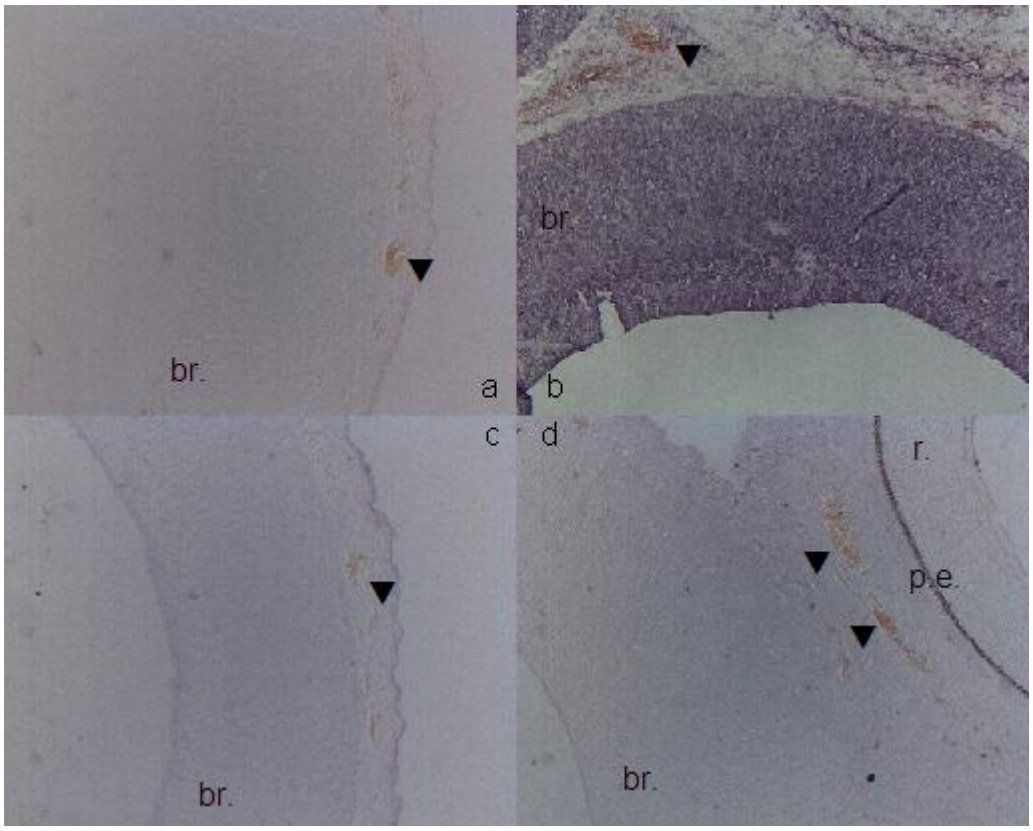

Figure 3.10 : IHC results with x5 magnification, showing the brain of *Gallus gallus* embryo. a) Negative control, b) Katanin p60, c) Katanin p80 d) Spastin. Brain is on the left side of sample d, retina is also seen on the right side of the same image. "br.", "r." and "p.e." represent midbrain, retina and pigment epithelium, respectively. On figure b, bottom area which seems as a thick line on the edge shows the katanin p60 expression in midbrain. The yellowish areas which are marked with black arrows show the blood tissues that do not show expression in any sample.

The expression levels on brain for four images; negative control was scored "0", katanin p60 was scored "+3", katanin p80 and spastin were inconclusive.

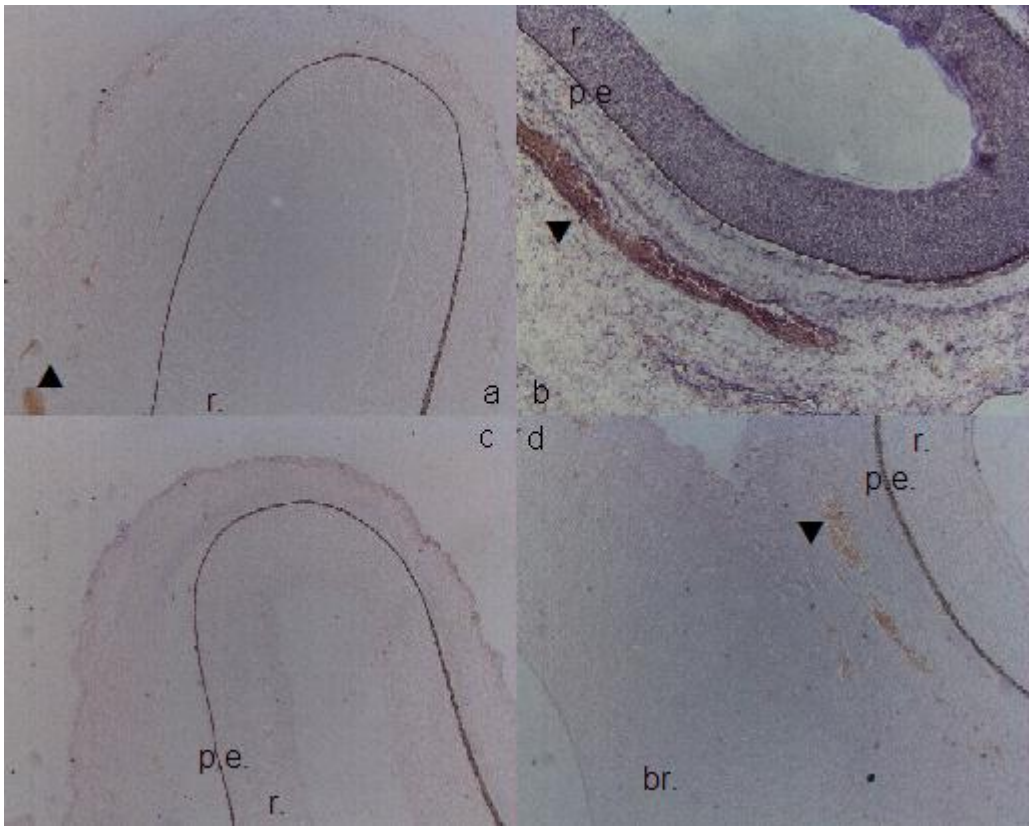

Figure 3.11 : IHC results with x5 magnification, showing the retina of *Gallus gallus* embryo. a) Negative control, b) Katanin p60, c) Katanin p80 d) Spastin. Retina is on the right side of sample d. Midbrain is also seem on the left side of the same image. "br.", "r." and "p.e." represent midbrain, retina and pigment epithelium, respectively. The darker, brownish lines in the middle show the retinal pigment epithelial layers. The inner sides of pigment epithelium show retina, sclera and choroid place on the outer side of pigment epithelium. On figure b, inner area which seems as a thick line on the upper side shows the katanin p60 expression in retina. The yellowish areas which are marked with black arrows show the blood tissues that do not show expression in any sample.

The expression levels on retina for four images; negative control was scored "0", katanin p60 was scored "+3", katanin p80 and spastin were inconclusive.

3.4. Comparison of the Results

In situ hybridization of katanin p60, katanin p80 and spastin proteins on *Gallus gallus* 5 and 7 day old embryos show that these three proteins are expressed strongly on spinal cord, notochord, somites, neural retina and brain. Expression of katanin p80 may be higher than the others, especially on notochord.

There are no results for katanin p80 and spastin with immunohistochemistry technique. Immunohistochemistry results of katanin p60 show expression on brain, retina, spinal cord and somites.

In summary, results of *in situ* hybrizidization and immunohistochemistry are consistent for katanin p60. Immunohistochesmistry studies neither support nor falsify the results of *in situ* hybrizidization for katanin p80 and spastin.

4. CONCLUSION

Microtubules are dynamic structures that undergo between growth and shrinkage phases continuously. In eukaryotic cells for many cellular functions such as cell division, cell differentiation, and nerve growth, dynamic instability of microtubules is essential. Microtubule reorganization is thought to be based on microtubule severing via evolutionary conserved AAA (ATPases Associated with diverse cellular Activities) family proteins katanin and spastin. Heterodimeric katanin protein has two subunits; katanin p60 and katanin p80 which are named according to their molecular weight. While katanin p60 has microtubule severing activity with ATP hydrolysis, katanin p80 directs and regulates the protein activity. Previous studies show that two subunits are found with different concentrations in different tissues even in different sites of a neuron. Another microtubule severing protein spastin, which structurely likes to katanin p60, is related with a neurodegenerative disease, hereditary spastic paraplegia.

In this project, *in situ* hybridization and immunohistochemistry techniques were applied to determine the expression of microtubule severing katanin p60, katanin p80 and spastin proteins in *Gallus gallus* embryos. *In situ* hybridization results show that katanin and spastin are expressed in all tissues except blood tissues. However, their expressions are much more higher on spinal cord, somites, notochord, brain and retina rather than the other tissues. Since their expression patterns are similar, it can be thought as their functions do not externalize each other. In fact, they may work cooperatively. On the other hand, despite the similar expression patterns on the samples, katanin p80 shows a better colour contrast on tissues. This makes think that expression of katanin p80 is higher than the others. The immunohistochemistry experiment supports the results of *in situ* hybridization for katanin p60, while there is no result for katanin p80 and spastin. Expression results were summarized on the following table.

Table 4.1 : Scoring of Expression Levels According to the ISH and IHC Results.

Technique	Tissue	Katanin p60	Katanin p80	Spastin
ISH	Spinal cord	+2	+3	+1
	Somite	+2	+3	+1
	Notochord	+1	+3	+0
	Brain	+2	+3	+1
	Retina	+3	+2	+2
IHC	Spinal Cord	+2	-	-
	Somite	+2	-	-
	Brain	+3	-	-
	Retina	+3	-	-

Since neurons are extremely differentiated cells, neurodegeneration of these cells leads irreversible damages on body. In order to better understand the neurodegenerative disorders like Alzheimer's and find a way for neuron's regeneration, it is very important to understand microtubule reorganization mechanism in nerve cells. Since reorganization of the cell is thought to be as a result of microtubule severing function by spastin and katanin, it is important to further analyze both expression, localization, function and regulation of these proteins.

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APPENDICES

APPENDIX A : List of Laboratory Equipments

APPENDIX B : List of Chemicals and KITs

APPENDIX C : Chicken katanin p60 Subunit

APPENDIX D : Chicken Katanin p80 Subunit

APPENDIX E : Chicken Spastin

APPENDIX A

Laboratory Equipments

- Flow Hoods (Waldner mc6, Özge, Faster BH-EN)
- Autoclave (Tomy SX-700E)
- Ice Machine (Scotsman AF10)
- Weighing Devices (Precisa XB 22A, Precisa BJ 610C)
- Microvave Oven (Arçelik MD582)
- Electrophoresis System (Wealtec Elite 300)
- Gel Screening System (Biolab UV Transulliminator)
- Spectrophotometer (Shimadzu UV 1601)
- Waterbath (Memmert)
- Egg Incubator (Brinsea Octagon 20DX)
- Magnetic Stirrer (Labworld 00.158232)
- pH meter (Inolab pH Level1 WTW)
- Cryostat (Leica CM1900 UV)
- Superfrost Slides (Menzel Gl.)
- Coverslips (Isotherm)
- Vortex (Heidolph Reax Top)
- Incubator (Memmert, Nüve EN400)
- Shaker (Heidolph Duomax 2030)
- Light Microscope (Leica DM2500 ,Olympus BX60)
- Block Heater (Biosan, Bio TDB-100)
- Tubes (BD Falcon Biosciences, Eppendorf)
- Micropipettes (Eppendorf Research, Gilson Pipetman)
- Pipet tips (Finntip, Eppendorf Research)

APPENDIX B

Chemicals and KITs

- DEPC (AppliChem, A0881)
- H_2O_2 (Merck, 108600)
- EDTA (Riedel-de Haen, 27285)
- NaOH (Riedel-de Haen, 06203)
- Tris (Amresco, 1076B049)
- Acetic acid (Riedel-de Haen, 27225)
- Agarose low EEO (AppliChem, A2114)
- EtBr (Amresco)
- RNA Loading Dye (Fermentas, #R0641)
- RNA Ladder Marker (Fermentas, #SM1821)
- PBS Tablets (Amresco, E404)
- Paraformaldehyde (Aldrich, S17977-315)
- Sucrose (Amresco, 1235B49)
- NaCl (Carlo Erba, 368257)
- Sodium citrate dihydrate (Merck, 106431)
- HCl (Merck, 100314)
- Ethanol (Tekkim, 200650.02500)
- O.C.T. – Tissue Freezing Medium (Leica - Jung, 14020108926)
- Gelatin (Gibco, 152-0221)
- Mixed Bed Resin (Sigma, M8032)
- Formamide (Promega, H5052)
- Dextran sulfate (Amresco, 0198)
- Denhardt’s Solution (Invitrogen, 750018)
- Salmon Sperm DNA (Invitrogen, 15632011)
- Anti-DIG AP, Fab Fragments (Roche, 11 093 274 910)
- BSA (Sigma, 49H0721)
- NBT/BCIP (Roche, 11 681 451 001)

- Mounting Medium (Sigma, 017K2334)
- Tween 20 (Sigma, P9416)
- Goat serum (Biological Industries, 992377)
- Donkey serum (Sigma, D9663)
- Katanin p60 antibody (produced in Dr.Karabay's Lab.)
- Goat anti-mouse IgG-AP (Santa Cruz, sc-2008)
- Katanin p80 B1 (C-12) Antibody (Santa Cruz, sc-107229)
- Donkey anti-goat IgG-AP (Santa Cruz, sc-2022)
- Spastin antibody (Abcam, ab35100)
- Goat anti-rabbit IgG-AP (Santa Cruz, sc-2007)
- DIG RNA Labeling Kit (SP6/T7) (Roche, 11 175 025 910)

APPENDIX C

Chicken Katanin p60 Subunit

DEFINITION : *Gallus gallus* p60 katanin mRNA, complete cds.

ACCESSION : DQ486889

SOURCE : *Gallus gallus* (chicken)

ORGANISM : *Gallus gallus*

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Archosauria; Dinosauria; Saurischia; Theropoda; Coelurosauria; Aves; Neognathae; Galliformes; Phasianidae; Phasianinae; Gallus.

AUTHORS : Korulu,S., Yildiz,A., Baas,P.W. and Karabay,A.

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 121 tactctctaa gagatacgta cctgcagcag aaatggcaac aggtttggca ggagataagt
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 781 aaagctgtag caactgaatg caagactact tttttcaatg tttcttcttc cacacttacc
 841 tcaaaataca ggggagaatc tgagaaactt gttcgtttgc tctttgaaat ggctcgattt
 901 tatgccccga caaccatatt tattgatgaa atagactcta tctgtagtcg acggggaact
 961 tcagaagagc atgaggctag ccggcgtgtg aaagcagagc tgctagttca aatggatggt
1021 gttggaggag ccactgaaaa tgatgatcct tctaaaatgg tcatggtact tgcagctact
1081 aattttccct gggatattga tgaagcccta agaaggaggc tagaaaagag aatttacatt
1141 cctttaccat cagcaaaagg tagagaggaa ctcctaagaa taaatctgcg ggagctggaa
1201 ctggctgatg atgttgacct tgcaaatata gctgagaaaa tggagggtta ttcaggtgca
1261 gacattacca acgtatgcag agatgcatcg ctgatggcta tgagaaggcg cattgaaggt
1321 ttgacaccag aagagataag aaatctttct cgagatgaaa tgcacatgcc aacaactatg
1381 gaagactttg aaatagcttt aaagaaggtt tctaagtctg tatctgctgc ggacattgag
1441 aaatatgaga aatggatagt tgaatttgga tcatgctga
```

* Yellow labeled part shows the sequence that was chosen for the probe design.

APPENDIX D

Chicken Katanin p80 Subunit

DEFINITION : *Gallus gallus* brain p80 katanin mRNA, complete cds.

ACCESSION : DQ410670

SOURCE : *Gallus gallus* (chicken)

ORGANISM : *Gallus gallus*

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Archosauria; Dinosauria; Saurischia; Theropoda; Coelurosauria; Aves; Neognathae; Galliformes; Phasianidae; Phasianinae; Gallus.

AUTHORS : Yildiz, A., Baas, P.W. and Karabay, A.

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   1 atggcagcgg ccgtcgtcac caagacggcc tggaagctgc aggagatcgt agctcacagc
  61 agcaatgtgt cctcattagt cctggggaag agcacgggcc ggctgctggc aactggagga
 121 gatgactgtc gggtcaacgt gtggtcagtt aacaagccca actgcgtcat gagcttgaca
 181 ggccacacga cacccattga gagcctacag atcagtgcaa aggaagaact cattgttgca
 241 gggtcccagt cagggtccat tcgagtctgg gacctggaag ctgccaaaat tctccgtacg
 301 ttacttggtc acaaggcaaa catctgcagc cttgatttcc atccttacgg aagtttcgtg
 361 gcatctggct ctttagatac agacattaag ctctgggacg tacgaagaaa aggctgcatc
 421 ttcaagtata agagccacac acaagcagtg agatgtcttc ggtttagtcc tgatggcaag
 481 tggttggcct ctgctgccga tgatcacact gtgaagctgt gggatctgac tgctgggaag
 541 gtaatgtttg agtttacagg acattctggc ccagtcaacg tcgttgaatt ccatcccagt
 601 gaataccttt tggcttctgg cagctctgac aggaccattc gtttctggga cttggagaag
 661 tttcacgttg tgagctgtat tgaagaggag gctactcctg tcaggtgtat tcttttcaac
 721 ccagatggct gctgcttgta tggtggcttc caggattctc tgcgtgtgta cggctgggag
 781 ccagagcgct gtttcgatgt ggtcgtagtg aactggggaa aagtagctga cttatctgtc
 841 tgccacaacc agctgatagg agtttccttt gcacaaagca cagtctcttc ctttgttgtg
 901 gatctcagca gagtcaccaa gtcaggttca gttcctcatg ggctgctcag gaacaacgag
 961 cttctggctc agcccactcc cacagggtcc tcccttcgtc gcagctatga cagaccctca
1021 actagctgca gcaagcctca gagagtgaag cacagttcag agagcgagag gcgcaatccc
1081 agcagtgaag aggaccggga tgagaaggaa tccaaggctg agatccagaa cccagaggat
1141 tacaaagaga tcttccagcc caggaatgcc atctctcgaa ctcctcctca tatcaatgag
1201 ccctttccag ccccccccaga ggatgagccc ataactgcaa aggaagcagt gaagcctaac
1261 caacctgtgg aagtccagac cccgctgcca acgcaagagc ttcctgagac atttcagagg
1321 ccaccaattg cttcctcaac tcctatgccc agagcagagc catcagtcat tcctgcagcc
1381 aggaacgagc ccattggcct gaaagcctct gacttcctac cagctctgaa aaaccaaagc
1441 caggctgaac tcacggatga agaaatcatg tcccagatca ggaaaggcca caagactgtg
1501 tgcatggtgc tcaccagccg ccacaagaat ctggacactg tgagggctgt atggagcacc
1561 agtgacatga agaactctgt ggacgctgcg gtagcaacca acgacctgtc tgttgttgtg
1621 gacctcttga acattgtcaa ccaaactgca tctctctgga agttggattt atgcaccgta
1681 gttctgcctc aaatagaaaa acttctccaa agtaaatatg aaagttatgt gcaaacgggc
1741 tgcacctcct tgaaactcat ccttcagaga ttcctgccac tgatcaccga catacttgct
1801 gcaccacctt ctgttggagt ggacatcacc agagaggaga ggctccataa atgcaggctg
1861 tgctacaagc agctgaaaaa catcagcaac attgtcaaga acaaatccgg gctcagcggc
1921 cgccacggta gtgccttcag agaactgcat ctcctcatgg ctgtgctgga gtga
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* Yellow labeled part shows the sequence that was chosen for the probe design.

APPENDIX E

Chicken Spastin

DEFINITION : *Gallus gallus* neuronal spastin mRNA, complete cds, alternatively spliced

ACCESSION : EU849600

SOURCE : *Gallus gallus* (chicken)

ORGANISM : *Gallus gallus*
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Archosauria; Dinosauria; Saurischia; Theropoda; Coelurosauria; Aves; Neognathae; Galliformes; Phasianidae; Phasianinae; Gallus.

AUTHORS : Karabay,A. and Akbalik,G.

```
   1 atggccgcca agagcagcag ggccggcgac gcgcccgagc ccggcggggc ggccgagcgg
  61 gtgcgggcgt gccacaagcg ggccttcgag tgcatctcca tggcgctgcg catcgacgag
 121 gacgagagag caggacaaaa ggaacaagct gttgaatggt ataagaaagg aattgaagag
 181 ctggaaagag gaatagctgt cttagttgtt ggtcaaggtg atcagtgtga acgggctcgg
 241 cgtctgcagt ctaaaatgat gaccaatttg gcaatggcca aggatcgctt gcagctttta
 301 gaaagtggag cagttccaaa aaagaaggat cccttaacac acacaagtaa ttcccttccg
 361 cgttcaaaaa cagttgcaaa aactggatcc acaggccttt caggccacca tagaacacct
 421 agctacagtg ggatatcaac tgcttctgtg tctaggccag cagcaaaccc tgcaacttca
 481 actcacaagg ctgctcctaa aaacagcaga acaaataagc cttctactcc tacccctgct
 541 gctcggaaga agaaagacac gaaagtattt agaaacgtgg acagtaatct tgctaatctt
 601 atcctgaatg aaattgttga tagtgggcca gctgtcaaat ttgatgatat tgcagggcag
 661 gagctggcta aacaagcttt gcaagaaatt gttatcctcc cttctcttag acctgagtta
 721 tttacaggac ttagagctcc tgcacgtgga ttgttgctct ttggcccacc aggaaatggg
 781 aagacaatgc tggccaaagc agttgctgca gaatcaaatg ctactttctt caatatcagc
 841 gctgcaagcc taacttcaaa atacgtgggt gagggtgaga aattggtgcg tgctctattt
 901 gcagtagcca gagaactgca gccttctata atttttattg atgaagttga tagccttttg
 961 tgtgaaagac gagaaggtga acatgatgct agtaggcgtc taaaaacaga attttttaata
1021 gaatttgatg gtgtgcagtc ttctggagag gacagaatac ttgtgatggg agcaacaaac
1081 aggccacagg agcttgatga tgctgttctc agacgattca ccaaacgggt atatgtatct
1141 ttaccaaatg aggaaacaag attgattttg ctaaaaaatc ttctaagcaa acaaggaagt
1201 ccgttgaccc aaaaagagtt ggcacagcta gcgagaatga cagatggata ctctggaagt
1261 gatttaactg catcagtaaa ggatgcagca ctgggtccta tccgagaact aaaaccagaa
1321 caggtgaaga acatgtctgc cagtgagatg agaaatatta aattatcaga tttcactgag
1381 tctttgaaga agataaagcg tagtttgagc cctcagacac tggaagcata cattcgttgg
1441 aacaaggact ttggagatac cacggtgtga
```

* Yellow labeled part shows the sequence that was chosen for the probe design.

CURRICULUM VITAE

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